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Polymer sieving matrices in microanalytical electrophoresis

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Microfluidic design has advanced existing protein separation capabilities and supported novel assays. Key metrics for successful protein separations include fast, robust, and sensitive analysis of complex mixtures of bio-macromolecules. Attaining high separation resolution is a chief concern. Here we review recent advances in polymer-based electrophoresis sieving materials that are impacting microfluidic bioanalytical applications. Looking forward, we comment on unmet needs for advanced separation media in micro-to-nanoscale devices.

1. Introduction

Polyacrylamide slab-gel electrophoresis has been a workhorse protein analysis tool for over four decades. In polyacrylamide gel electrophoresis (PAGE), the hydrogel acts as a molecular sieving matrix underpinning size-based separation of proteins, thus enabling more straightforward protein identification than conferred by determining the charge-to-mass ratio.¹ To accurately determine protein molecular mass, proteins are treated with the surfactant sodium dodecyl sulphate (SDS),^{2,3} making slab-gel SDS-PAGE a dominant protein analysis tool.⁴ Polyacrylamide gel is also an anti-convective medium yielding sharp separated protein zones with minimal convective dispersion.¹ However, slab-gel electrophoresis is far from ideal; the tool suffers from: appreciable sample consumption, significant heat generation, poor reproducibility, semi-quantitative results due to run-to-run variability, laborious manual handling, and cumbersome post-separation analysis relying on gel staining and washing.¹

To overcome the limitations of slab-gel electrophoresis, capillary gel electrophoresis was introduced.^{5,6} Electrophoresis in small bore glass capillaries (diameter: 10–300 μm) offers performance metrics that can be superior to slab-gels including: small sample consumption, fast separation speeds enabled by high applied electric fields,⁷ UV-⁷ or LIF (laser-induced fluorescence)-based on-line detection,¹ a capacity for quantitative analysis, efficient cooling by effective heat dissipation,⁷ and

automated operation.¹ While slab-gels separate multiple protein samples per gel, capillaries can be arrayed for the parallel protein analysis.⁷ Introduction of capillary-filling gels (polyacrylamide, agarose) yielded a hybrid separation technique offering fast size determination with low peak dispersion.^{5,6}

Crosslinked gels were supplanted by “replaceable” non-crosslinked water-soluble polymers, such as linear polyacrylamide (LPA).⁸ Replaceable gels gained popularity, as capillaries filled with crosslinked gels cannot be readily replenished by pressure-driven flushing of the channel. Refreshing the sieving media allows reuse of a channel, even if the separation matrix has deteriorated. Specifically, complex biological samples often clog the sieving matrix and sometimes bubbles form because of Joule heating in the channel. Polymer solutions, although not crosslinked, are “entangled” to form the dynamic polymer network acting as a molecular sieve.⁷ A consideration of non-crosslinked polymer separation media is the need for wall surface coatings to minimize EOF (electroosmotic flow) and analyte adsorption; both are sources of dispersion.

Electrophoretic separation conducted in microdevices has been a dominant trend in modern analytical chemistry owing to the possibility of mass fabrication by exploiting the mature microfabrication techniques derived from the semiconductor industry. Further interest stems from the capability of integrating pre- and post-separation analytical steps for streamlined protein analysis (*e.g.*, the lab-on-a-chip concept) and the possibility of massive parallelization.^{9–11} Analytical instrumentation has been adopted from capillary electrophoresis (CE) and adapted to microfluidic electrophoresis (*e.g.*, high-voltage power supply, LIF detection, and crosslinked or non-crosslinked sieving media). For microfluidic gel electrophoresis, linear polymers have been used extensively due to the benefits of replaceable gels, while crosslinked gels have only recently drawn attention from the analytical community. In particular, *in situ* polymerized crosslinked polyacrylamide gels are

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compatible with photolithographic fabrication and provide unique advantages including the possibilities of: integrating multiple functionalities into a single chip and extremely short separation distances which support large-scale integration of electrophoresis in a single microdevice.

Ideally, sieving matrices for electrophoresis separations would offer: (1) a uniform pore distribution for reproducible separation, (2) no adsorption to analytes and low-to-no background signal, (3) compatibility to an appropriate detection method [e.g., fluorescence detection, mass spectrometry or surface enhanced Raman spectroscopy (SERS)], and (4) robust operation (e.g., resistance to high electric fields, and chemical decomposition). In addition to these essentials are properties specific to each application or format.

In this review, we focus on polymer sieving matrices prepared inside channels or microfluidic devices for advanced proteomic applications. We have intentionally omitted developments in separation media developed primarily for off-chip analytical operations. Instead of considering historical rarely used separation media, we emphasize recent novel approaches and materials. We summarize separation matrix properties, common or particularly noteworthy separation conditions, and interesting applications using these functional materials.

2. Microanalytical separation mechanisms in crosslinked gels and entangled polymer solutions

The native conformation of protein species spans a staggering range of molecular masses, geometric shapes, and surface charges. Electrophoresis separations of native proteins arise from a differential mobility, μ , between species of interest. The electrophoretic mobility is a function of the charge-to-mass ratio of each protein species. The mobility difference can be enhanced using a molecular sieving matrix (as compared to free solution), so that protein bands migrate as sharp zones, resolved from neighbouring species.¹ Denatured proteins can unfold and adopt a rod-like shape.¹² When proteins complex with a detergent like SDS, all proteins acquire the similar charge-to-mass ratio and, thus, the near identical electrophoretic mobility in background buffer.¹³ Thus, in free solution, the species would not resolve from each other during electrophoresis. In contrast, during electromigrating through a sieving matrix, the SDS-treated proteins interact with the molecular sieve yielding different electrophoretic mobilities for the different species. The mobility in this case depends on molecular mass, which affords size-based protein separations and ultimately allows molecular-mass determination. Because of these advantages, electrophoresis of proteins is commonly performed using water-soluble molecular-sieving polymer networks.

Gel-based sieving matrix can be largely categorized into crosslinked gels and non-crosslinked gels. Crosslinked gels have a well-defined gel pore structure (Fig. 1a).¹² The pore size ξ strongly depends on total monomer (%T) and crosslinker concentration (%C, weight percentage of crosslinker in total

acrylamide content of a gel). Once polymerized, the pore structure of a crosslinked gel is static ("chemical gel", Fig. 1a). In comparison, an entangled polymer solution supports transient gel fibre networks. The most widely used crosslinked sieving matrix is polyacrylamide gel. Because of extremely high viscosity, the gels are usually polymerized inside the capillary^{5,6,14} or microchannels.^{15–24}

Non-crosslinked, slightly branched or linear hydrophilic polymer chains are entangled in solution and form a polymer network.¹ In dilute solution, the polymer chains are hydrodynamically isolated (Fig. 1b). As the polymer concentration C increases above the "overlap threshold concentration" C^* , the polymer chains interact more frequently and become entangled to form a network. In contrast to the crosslinked gel which utilizes chemical interactions between chains, the chain interactions in an entangled network are physical. Thus, pores of the entangled network are dynamic and transient. The links between polymer chains can be reversibly broken by thermal motion, diffusion, electrokinetic migration and entrainment by migrating biopolymers. Soon after breakage, links are reformed owing to physical interactions.²⁵ The pore size of the entangled network is a function of the radius of gyration (size, in a loose sense) of a polymer chain and decreases with polymer concentration. Due to the dynamic nature, the relaxation times of the entangled polymer mesh (*i.e.*, lifetime) relative to the residence time of migrating biopolymers are important for sieving action.²⁶ The relaxation time should be orders of magnitude larger than the residence time. As a result, separating slow-moving (*i.e.*, long residence time) high-molecular-mass analytes can prove challenging.²⁶

Here we briefly introduce mechanisms of proteins separation, which can be equally applied to separation of DNA, another biopolymer.^{7,12} Three different separation mechanisms describe electrophoretic migration through a polymer network, depending on shape and size of a protein relative to the pore size and applied electric field (Fig. 2).^{1,12} In the Ogston model, a protein is assumed to be a rigid sphere and the average pore size ξ is comparable to the hydrodynamic radius of protein R_{gp} . The electrophoretic mobility μ of the protein is given by:

$$\mu = \mu_0 \exp[-Cb(R_{gp} + r)^2] \quad (1)$$

where μ_0 is the free-solution mobility, C is the sieving-polymer concentration, r is the radius of polymer fibre, and b is a constant. Here log of the protein mobility is a linear function of log of the protein molecular mass (Fig. 2b). The Ferguson relation is derived from the Ogston model, assuming that electric field does not affect R_{gp} and r is much smaller than R_{gp} .²⁸

$$\mu = \mu_0 \exp[-K_R C] \quad (2)$$

Using the Ferguson relationship eqn (2), the size of SDS-treated proteins or native proteins can be estimated. The Ogston model was used to describe SDS-treated protein migration in a crosslinked gel.^{28,29}

Fig. 1 (a) Crosslinked polymer network of average pore size ξ and solute of size R_g . Reprinted with permission from ref. 26, © 1993 Elsevier. (b) Flexible polymers in solution. (1) Polymer chains are hydrodynamically isolated in a dilute polymer solution ($C < C^*$); (2) polymer chains touch each other at the overlap concentration ($C = C^*$); (3) polymer chains are entangled and form a dynamic network in a semi-dilute polymer solution ($C > C^*$). Reprinted with permission from ref. 27, © 2003 Wiley InterScience.

Fig. 2 (a) Mechanisms of electrophoretic biopolymer migration in a polymer network: (1) the Ogston model; (2) the reptation without stretching model; (3) the reptation with stretching model, reprinted with permission from ref. 27, © 2003 Wiley InterScience. (b) The relationship between the logarithm of electrophoretic mobility and the logarithm of biomolecule molecular mass, M_r . Reprinted with permission from ref. 1, © 1996 Wiley InterScience.

The reptation model describes the migration of large bi-macromolecules as flexible chains. Using snake-like motion (*i.e.*, reptation), biopolymers can migrate through a mesh with pore-sizes significantly smaller than the size of solute. In this case, the mobility is inversely proportional to the molecular mass (M_r) of the biomolecule.

$$\mu \sim \frac{1}{M_r} \quad (3)$$

The reptation with stretching model describes the situation where biomolecules are stretched under the electric field and migrate through the pores with the snake-like motion. The mobility is given by:

$$\mu \sim \left(\frac{1}{M_r} + bE^2 \right) \quad (4)$$

where E is the applied electric field and b is the constant which is a function of the pore size. Guttman studied capillary electrophoresis of SDS-treated protein in polyethylene oxide (PEO) solution to see which model suitably describes protein migration in a dynamic polymer network.¹² He observed no linear relationship between $\log M_r$ and $\log \mu$, suggesting that the Ogston model did not apply. Also, migration behaviour of the SDS-protein complexes relied on polymer chain length [b in eqn (4)] and applied electric field [E in eqn (4)], indicating that the

reptation with stretching model may suitably describe the SDS-treated protein migration through polymer solution. For more detail on mechanisms, the reader is directed to articles by Viovy,^{25,27} Soane^{26,30} and Heller.⁷

Using these fundamental principles, researchers have explored a wide space of molecular sieving materials for protein electrophoresis. Spanning from linear polyacrylamide solutions used in commercial capillary and microchip systems, to novel carbon-nanotube-based sieving matrices at the cutting edge of research. While not exhaustive, we provide an overview of research involving molecular sieving materials relevant to microfluidic electrophoresis systems, with a summary of detailed materials given in Table 1.

3. Replaceable, water-soluble polymer solutions as separation matrices

In the first paper on capillary gel electrophoresis (CGE) by Hjertén, agarose and polyacrylamide (PA) gels were employed as sieving matrices.⁵ *In situ* polymerization inside the capillary presented a natural extension of supporting-media preparation in conventional slab-gel electrophoresis systems² to the newer CE format. Having “static” molecular sieving pores and covalent linkage to the capillary surface, crosslinked gels afford excellent

Table 1 Molecular sieving materials for protein electrophoresis

Material	Chemical structure	Advantages	Disadvantages	Analytes	Polymer concentration/molecular mass (or viscosity)	Separation efficiency [plates per m]/separation distance	Ref.
LPA (linear polyacrylamide)		<ul style="list-style-type: none"> • Most widely used • Excellent separation resolution • Optically transparent 	<ul style="list-style-type: none"> • High viscosity • Hydrolysis in alkaline condition • Strong UV absorbance • Fluorescence background when concentrated • No surface coating capacity • Neurotoxicity 	Serum glycoprotein, N-glycans	4%/9 MDa	—	31
				Protein standard (21.5–116.0 kDa)	5%/0.6–1 MDa	0.5–5.3 × 10 ⁵ /5 mm	32
				Protein standard (21.5–116.0 kDa)	5%/0.6–1 MDa	9–23 × 10 ⁵ /5 mm	33
				Protein standard (18–116 kDa)	3%/2.16 MDa	—	34
				CA, BSA	12%/1.5 × 10 ⁴ cP at 12% 2%/in situ polymerized	—	35
				Thrombin/thrombin–aptamer complex	1–2 × 10 ⁴ /25 mm	—	36
PDMA (polydimethylacrylamide)		<ul style="list-style-type: none"> • Low viscosity • Resistance to alkaline hydrolysis • Surface coating capacity 	<ul style="list-style-type: none"> • Less resolution than LPA • More hydrophobic than LPA 	Protein standard (14–205 kDa)	3.25%/proprietary	~10 ⁷ /14 mm	37
				Protein standard (17–66 kDa)	5%/200 kDa	9 × 10 ⁵ /30 mm	38
PVP (polyvinylpyrrolidone)		<ul style="list-style-type: none"> • Low viscosity • Surface coating capacity 	<ul style="list-style-type: none"> • Less resolution than LPA • More hydrophobic than LPA 	CA isoforms	1.8%/0.36–1.3 MDa	—	39
Dextran		<ul style="list-style-type: none"> • Low viscosity • Low UV absorbance • Separation resolution as high as LPA 	<ul style="list-style-type: none"> • Weak surface coating capacity⁴⁰ • Heat required to improve separation⁴¹ 	Protein standard (14.4–29.0 kDa)	6%/6 cP	4.4–4.6 × 10 ⁵ /30 mm	42
				α-Lactalbumin, β-lactoglobulin, BSA	10%/425–575 kDa	1.8 × 10 ⁵ /3 mm	43
Pullulan		<ul style="list-style-type: none"> • Low viscosity • Low UV absorbance • Separation resolution comparable to PAGE 	<ul style="list-style-type: none"> • Weak surface coating capacity (additives required)^{44,45} 	Protein standard (6 proteins, not specified)	15%/—	—	46

Table 1 (Contd.)

Material	Chemical structure	Advantages	Disadvantages	Analytes	Polymer concentration/molecular mass (or viscosity)	Separation efficiency [plates per m]/separation distance	Ref.
MC (methyl-cellulose)		<ul style="list-style-type: none">• Surface coating capacity (especially for plastic surface)	<ul style="list-style-type: none">• Relatively hydrophobic• Detergent required to improve separation⁴⁷	TI, BSA, amyloglucosidase/BSA/BSA-antibody complex	2%/3500–5600 cP at 2%	3.6 × 10 ⁵ /30 mm	47
HPC (hydroxy-propylcellulose)		<ul style="list-style-type: none">• Thermo-responsive (viscosity changing) polymer• Excellent dynamic surface coating	<ul style="list-style-type: none">• Incompatible with dynamic labelling dye⁴⁸• Require higher concentration for separation than hydroxyethylcellulose does⁴⁹	Lipoproteins HDL, sdLDL, ILDL	0.6%/80 kDa	1.5 × 10 ⁶ /42.5 mm	50
HEC (hydroxy-ethylcellulose)		<ul style="list-style-type: none">• Robust entangled network at low concentration^{49,51}• Low viscosity	<ul style="list-style-type: none">• Purification required• Batch-to-batch variability	Fluorescence proteins RPE, BPE and GFP BSA, GFP isoforms	1%/250 kDa 0.2%/90 kDa	—, (*IEF assay) —, (*IEF assay)	52 53
HPMC (hydroxypropyl-methylcellulose)		<ul style="list-style-type: none">• Low viscosity• Surface coating capacity	<ul style="list-style-type: none">• Additives required to improve separation⁵⁴	Protein standard (14–205 kDa) TI, BSA Lipoproteins HDL, LDL, VLDL	1%/— 0.2%/4000 cP at 2% 1%/4000 cP at 2%	—, (*ITP assay) 8.2 × 10 ⁵ , 30 mm —	55 56 57
PEO-PPO-PEO (polyethylene oxide-polypropylene oxide-polyethylene oxide)		<ul style="list-style-type: none">• Thermo-responsive character• Adjustable pore structure	<ul style="list-style-type: none">• Limited to DNA applications• Difficult to replenish	Oligonucleotide standard (8–32 base)	30%	—	58
PEO (polyethylene oxide)		<ul style="list-style-type: none">• Surface coating capacity• Relatively low viscosity• Low UV absorbance	<ul style="list-style-type: none">• Hydrolysis in alkaline condition• More hydrophobic than LPA• Separation degraded at high temperature⁶⁰	Protein standard (9–116 kDa) (1) Actin, OVA, protein A (2) BSA, helix pomatia lectin	Proprietary (Beckman 14-200) Proprietary (Beckman 14-200)	2–4 × 10 ⁵ /45 mm 1.6 × 10 ⁶ /30 mm	61 62

Table 1 (Contd.)

Material	Chemical structure	Advantages	Disadvantages	Analytes	Polymer concentration/ molecular mass (or viscosity)	Separation efficiency [plates per m]/separation distance	Ref.
Polyacrylamide		<ul style="list-style-type: none"> • Nonreactive with fluorogenic labelling reagent⁵⁹ • Separation comparable with PAGE • Low interaction to DNA or proteins • Verified for long time • Variety of applications • Diverse chemical modification • Capable of photopatterning 	<ul style="list-style-type: none"> • Gel breakdown • Difficult to replenish • Heterogeneity at nanoscale 	(3) Concanavalin A, lectin peanut agglutinin <i>E. coli</i> cell lysate	2%/600 kDa	—	60
				Protein standard (20–116 kDa) Protein standard (12–67 kDa)	3.5% to 10% <i>T</i> gradient gel 3.6% <i>T</i> 0.4% <i>C</i>	3.3 × 10 ² /3 mm 2880 (Peak capacity defined in ref. 63) 3.0 × 10 ³ /0.5 mm	16 63 64
				Protein standard (21–67 kDa)/human seminal fluid Protein standard (21–116 kDa) human sera Protein standard (21–67 kDa)	6% <i>T</i> 2.5% <i>C</i> 7.5% <i>T</i> 2.5% <i>C</i> 10% to 20% <i>T</i> 3.33% <i>C</i> (free-standing gel)	1.3 × 10 ³ , 4.4 × 10 ³ (gradient gel)/1 mm	23 65
Polymethacrylate		<ul style="list-style-type: none"> • Low interaction to DNA or proteins • Biocompatible • Ready chemical modification • Capable of photopatterning 	<ul style="list-style-type: none"> • Gel breakdown • Difficult to refresh • Heterogeneity at nanoscale • Potential non-specific adsorption to basic proteins 	Protein standard (18–240 kDa) Peptide mixture	3% 40%	— —	66 67
PNIPAAm [poly(<i>N</i> -isopropylacrylamide)]		<ul style="list-style-type: none"> • Superior thermo-responsive character 	<ul style="list-style-type: none"> • More hydrophobic than polyacrylamide • Protein adsorption 	Protein standard (14.4–97.4 kDa)	1.1% <i>T</i> 0.025% <i>C</i>	—	68
PS- <i>b</i> -P4VP [polystyrene- <i>b</i> -poly(4-vinylpyridine)]		<ul style="list-style-type: none"> • pH-responsive character • Low interaction with proteins compared to other pH-responsive polymer 	<ul style="list-style-type: none"> • Difficult to replenish 	BSA and immunoglobulin-γ	15%	—	69

resolving power and complete elimination of convection. However, the crosslinked gels in CGE have several critical limitations. Bubbles often form inside the capillary because of the gel shrinkage during polymerization,^{7,70} alkaline hydrolysis,⁷ and high temperature and/or high current during electrophoresis.⁵⁴ Bubble generation can break the electrical circuit and thus terminate electrophoresis. Electrophoretic sample injection is common in crosslinked gels, and, in some configurations, uniform loading of sample of different electrophoretic mobilities is difficult.³⁵ Further, clogs arise at the capillary entrance during sample injection.⁷ Residual high molecular mass analytes can be retained in the column, yielding carry-over to subsequent electrophoresis runs.^{1,7} Such issues have limited assay repeatability to the first few tens of runs.^{7,27,70}

To allow for a crosslinked PA gel that is prepared *ex situ* and then pressure-loaded into the capillary, efforts to lower the viscosity of the *ex situ* prepared PA gel have been made. One such effort focused on reducing the crosslinker (*i.e.*, Bis) concentration to 0.4%*C*, down from the more usual ~3%*C*.^{4,71} These replaceable crosslinked PA (rCPA) gels were injected into capillaries using pressures of 80 psi.⁴ The inner surface of the capillary is coated by *in situ* polymerization of PA to alleviate analyte adsorption. To our knowledge, rCPA has not been adopted by microfluidic protein electrophoresis.

Water-soluble non-crosslinked polymers were employed as alternatives to crosslinked gels. A wide range of polymers are used in both nucleic acid and protein separations. The separation mechanism of nucleic acids is similar to that of proteins, especially SDS-treated, linearized proteins.¹² The sieving matrices include polyacrylamide *e.g.*, LPA, polymers that are acrylamide derivatives (*e.g.*, PDMA), vinyl polymers (*e.g.*, PVP), polysaccharide derivatives (*e.g.*, dextran, pullulan, MC, HPC, HPMC, HEC), and polyether (*e.g.*, PEO). Above the critical concentration (*i.e.*, entanglement threshold *C**) the polymer forms dynamic pores by chain entanglement,⁷ which acts as a molecular sieve. The advantages of the uncrosslinked polymer solutions in CGE include: (1) facile gel preparation;⁷² (2) viscosity as low as 10 cP, which enables easy replacement of the degraded gels and thus allows multiple runs in one capillary (>100 runs);⁷³ (3) tuneable gel properties including modifying the polymer concentration, the addition of surfactants and surface coating reagents; and (4) long shelf life.^{72,74} Because of these attractive properties, numerous studies adapt the polymer solution used in CGE to microfluidic electrophoresis formats. One of the most commercially successfully microchip electrophoresis platforms (*i.e.*, Agilent Bioanalyzer 2100) employs a PDMA-based replaceable polymer solution.³⁷ For detailed reviews on replaceable polymer solution of biopolymer electrophoresis, the reader is directed to articles by Albarghouthi and Barron,⁷⁵ Guttman,⁵⁴ Hsieh *et al.*,⁷³ Mikšik *et al.*,⁷⁶ and Zhu *et al.*⁷⁰

One important consideration in replaceable polymer systems is column surface passivation. Owing to a surface functional groups or analyte newly adsorbed to the surface, significant EOF can be generated.⁷⁷ EOF can cause distortion and dispersion of protein bands, thus deteriorating separation performance. Also, proteins tend to adsorb to microchannel surfaces *via* various intermolecular forces, which yields the band broadening,

irreproducible separation, or even an inability to inject samples. As polymer substrates including PDMS (polydimethylsiloxane), PMMA [poly(methyl methacrylate)] and COC (cyclic olefin copolymer) become widely adopted in microfluidics,⁷⁸ analyte adsorption has become a critical issue for protein separations in polymeric microfluidic devices. While glass surfaces can be covalently coated with polymers (*e.g.*, PA) using popular bifunctional aminosilane reagents,⁵⁴ more complicated chemistries may be required for plastic surface passivation (*e.g.*, graft polymerization).⁷⁹ Covalent coating strategies involve laborious preparation steps, and the coatings may degrade after multiple electrophoresis runs. Therefore, non-covalent “dynamic coatings” are widely employed. The simple addition of coating reagents (usually water-soluble polymers) to the background buffer before electrophoresis can be sufficient. Dynamic coatings can be replenished by replacing the background buffer, so that run-to-run reproducibility is excellent.⁵⁴ Reagents for this purpose include polymers of acrylamide^{60,80} and acrylamide derivatives such as PDMA³⁴ and PHEA (poly-*N*-hydroxyethylacrylamide),⁸¹ polysaccharide and its derivatives such as dextran,⁸² HPMC,⁵⁶ MC,⁸³ HEC,^{53,84,85} HPC,⁸⁶ cationic HEC,⁸⁷ MHEC (methylhydroxyethylcellulose),³⁶ cationic starch derivatives⁸⁸ and other polymers such as PVP,⁸⁹ PVA (polyvinyl alcohol)⁹⁰ and PEG (polyethylene glycol).⁹¹ For detail, readers are directed to the recent reviews by Horvath and Dolník,⁸³ Dolník,⁹¹ Doherty *et al.*,⁸¹ and Belder and Ludwig.⁹²

Lastly, novel sieving matrices that offer complex properties are being more widely adopted. These include: thermally responsive polymers,⁹³ interpenetrating networks (IPN)⁹⁴ and carbon nanotube (CNT) modified polymers (see chapter 5 also for more detail).⁹⁵ While somewhat complicated to prepare, all of these polymers were intended to prevent analyte adsorption, facilitate polymer loading and replacement, and afford high-performance separations.

3.1. Linear polyacrylamide (LPA)

LPA is possibly one of the most widely used non-crosslinked separations matrices. The LPA offers excellent protein separation resolution^{4,75} and is transparent for optical detection. However, LPA is highly viscous (*i.e.*, ~260 000 cP for 9 MDa, 2% LPA⁷²) and thus gel loading into small bore columns requires high pressures. Fortunately, LPA is a non-Newtonian fluid with a viscosity that decreases with increasing shear rates (*i.e.*, 27 600 cP for a shear rate of 1.32 s⁻¹, 9 MDa, 2% LPA⁷²). Polyacrylamide can hydrolyse in alkaline condition,⁷ resulting in charge interactions between the LPA and protein analytes.²⁰ The LPA absorbs UV – especially below 230 nm (ref. 96) – resulting in high background signals for UV protein detection.¹³ Such background signal is not so critical for microfluidic protein electrophoresis, where fluorescence detection and not UV detection is dominant. On the other hand, the high fluorescence background signal of concentrated LPA can interfere when using fluorescence detection.⁷³ When LPA is used as a sieving medium, glass-surface passivation is usually required to suppress EOF and to prevent nonspecific protein adsorption.⁷⁵ The neurotoxicity of the acrylamide monomer is also a concern.⁹⁷

Callewaert *et al.* reported *N*-glycome profiling in human sera.³¹ The serum was electrophoretically analyzed for fibrosis follow-up and cirrhosis diagnosis using 11.5 cm-long microfluidic channels in an alumina-silicate glass chip. A high-molecular-mass LPA (~9 MDa) was prepared using inverse emulsion polymerization.⁷² The sieving matrix was a 4% LPA solution in 1× TTE buffer consisting of Tris [tris(hydroxymethyl)aminomethane], TAPS [N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid], EDTA (ethylenediaminetetraacetic acid) and 7 M urea. The chip surface was covalently coated with MC and dextran for suppression of EOF and solute adsorption.⁸² The microchip electrophoresis system reported separation resolution similar to that obtained using a commercial CE system (ABI377, Applied Biosystems).

Baba's research group has extensively studied linear polymer sieving matrices and coating reagents for protein electrophoresis in PMMA chips.^{32,33,39,42,47,56,57} The researchers reported SDS CGE of proteins on a PEG-coated commercial PMMA microfluidic chip.³² Here, a covalently attached PEG coating effectively suppressed protein adsorption. A ladder of proteins (21.5–116.0 kDa) was separated in a 5% LPA (0.6–1 MDa, in 0.1% SDS and 50 mM Tris–HCl buffer) in a short 3 mm separation length and fast 8 s separation time. LPA solutions of higher molecular mass and higher concentrations were reported as difficult to load into the chip. LPA solutions of lower molecular mass than the optimized solution did not support a fully resolved ladder. The same group also studied the dynamic surface coating properties of SDS on EOF in an otherwise uncoated PMMA chip.³³ The authors suggest that the SDS bound to the PMMA surface and induced EOF when an electric field was applied. Owing to EOF, separation of SDS–protein complexes was faster (~6 s) but a longer separation distance was necessary, compared to the PEG surface-coating conditions (both conditions used the same 5% LPA as a sieving matrix).³²

Root *et al.* used an acid-labile surfactant (ALS) to replace SDS for size-based protein electrophoresis, followed by mass spectrometry analysis.³⁴ ALS decomposes at low pH values, thus reducing interference with mass spectrometry analysis seen when employing SDS.⁸⁹ The glass microfluidic chip surface was coated with PHEA, PDMA, PVP, HPMC, or PEO and protein adsorption was evaluated. Surface coating with 0.1% PHEA yielded optimal protein separation efficiency. After 15 min of PHEA incubation, ALS-treated ladder proteins [β -lactoglobulin, carbonic anhydrase (CA), ovalbumin (OVA), bovine serum albumin (BSA), and β -galactosidase] were successfully separated using 3% LPA (2.16 MDa) sieving matrix prepared in 1× TTE (49 mM Tris, 49 mM TAPS, and 2 mM EDTA) + 0.5% ALS buffer. The separation efficiency and elution time were on par with SDS gel electrophoresis.

In Qi *et al.*'s work, a highly viscous *ex situ* polymerized 12% LPA (~1.5 × 10⁴ cP) was loaded only into the separation channel of a glass chip housing a double-T junction.³⁵ The remaining sample, buffer and buffer-waste channels were filled with 20 mM Tris–20 mM Tricine solution and no gel. All the channels were covalently coated with LPA¹⁴ for EOF and protein adsorption suppression. Sample loading was performed through a combination of electrokinetic injection and pressure

injection *via* the three open solution channels (no gels). BSA and CA were separated in <150 s and in 2 cm separation distance. The authors also demonstrated DNA separation in 2% HEC (~102 cP) using the same chip.

Lastly, Soper's group analyzed plasma-levels of thrombin using non-equilibrium affinity CE (ACE) in a PMMA microfluidic chip.³⁶ The microchannel was non-covalently coated with 5% MHEC for 10 min and a 2% LPA sieving gel prepared in 25 mM Tris–glycine buffer with 0.01% MHEC was loaded. Without the MHEC dynamic surface coating, the thrombin electromigration was not reproducible. Thrombin could not be repeatedly loaded into the separation channel because of EOF and solute adsorption. Also, the authors reported that without the LPA sieving gel (*i.e.*, free-solution electrophoresis), association between an affinity probe (aptamer) and thrombin was not observed. The authors speculated that a “cage effect” between the entangled LPA network and slow diffusion in the high viscosity background buffer improved association of aptamer to thrombin. The separation efficiency of 1–2 × 10⁴ plates per m was obtained.

3.2. Polydimethylacrylamide (PDMA)

With a viscosity much lower than that of LPA, PDMA was applied to DNA analysis *via* CE (75 cP for 6.5% 98 kDa PDMA).⁷⁵ Given favourable viscosity, lower channel-filling pressures and faster gel replacement cycles were supported. PDMA is also more resistant to alkaline hydrolysis than LPA. PDMA also mitigates analyte surface adsorption to a greater extent than LPA by strongly adsorbing to a silica surface because water is “poor” solvent for relatively hydrophobic PDMA compared to LPA.⁹⁷ No special conditioning such as HCl pre-treatment of the glass surface is required for PDMA dynamic surface coating. Nevertheless, separation resolution obtained with PDMA is not usually as good as that found using LPA as a sieving matrix. PDMA is more hydrophobic than the LPA, affecting protein separation performance (*e.g.*, interaction with fluorescent dye, band broadening, peak shifting).⁷⁵ One of the most widely used commercial microfluidic electrophoresis systems (*i.e.*, Agilent Bioanalyzer 2100) uses PDMA-based polymer as a sieving matrix.^{37,98} Standard proteins [lysozyme, soybean trypsin inhibitor (TI), CA, OVA, BSA, phosphorylase b, β -galactosidase, and myosin] have been separated in 40 s using background buffer consisting of 42 mM Tris, 120 mM Tricine, 0.25% SDS and 3.25% of the PDMA-based polymer. Separation efficiencies 1–2 orders of magnitude higher than SDS CGE (of 10⁷ theoretical plates per m) have been reported.³⁷

PDMS is a popular substrate material for microfluidics but is prone to protein surface adsorption due to its hydrophobicity. Wu *et al.* covalently coated the surface with epoxy-modified hydrophilic polymers including poly[DMA (dimethylacrylamide)-*co*-GMA (glycidylmethacrylate)], PVP-*g*-GMA and PVA-*g*-GMA.³⁸ The permanent surface coating reduced protein adsorption to <10% of the adsorption observed on native PDMS surfaces, with EOF suppressed significantly. 5% PDMA in 100 mM Tris–TAPS was used as a sieving matrix and a standard protein ladder (17–66 kDa) was separated in <20 s in a

separation distance of 3 cm. The authors postulated that the PDMA surface coating was more stable than non-covalent protein (*e.g.*, IgG, neutravidin) or dextran coatings. Column efficiencies of 9×10^5 plate per m were observed.

3.3. Polyvinylpyrrolidone (PVP)

The viscosity of PVP solutions is unusually low (3 cP at 1% concentration), thus pressure-driven flushing of this polymer solution is relatively easy.⁷⁵ In addition, PVP reduces the EOF. Thus, PVP has been used in DNA separation in capillary formats⁹⁹ (5–5.5% concentration) and microfluidic formats (2% concentration).⁷⁴ Baba's group used this low-viscosity polymer for separation of CA (carbonic anhydrase) isoforms.³⁹ Interestingly, the study employed a divalent metal ion Mg^{2+} as a "dynamic crosslinking reagent" for the PVP chains. 0–5 mM of Mg^{2+} was added to 1.8% PVP solutions in 89 mM Tris-borate and 2 mM EDTA. The authors observed increases in separation efficiency of the protein isoforms and speculated that separation performance was improved owing to decreases in the polymer screening length (*i.e.*, mesh size) and increases in the hydrodynamic radius of PVP molecules by the dynamic cross-linking (Fig. 3).

3.4. Polysaccharide derivatives

Linear (*e.g.*, cellulose) or slightly branched (*e.g.*, pullulan, dextran) polysaccharides are popular sieving matrices for biopolymers.^{70,75,76} Especially, a variety of natural or synthetic celluloses were employed.^{40,100} One important benefit of these linear polymers in conventional CE is low UV absorbance (especially 214 nm) compared to LPA⁷⁰ thus allowing on-line UV detection of separated proteins without labor-intensive staining/destaining processes. However, the analytical sensitivity of UV absorbance is orders of magnitude poorer than limits offered by LIF detection. Therefore, low UV absorbance of the polysaccharides is not normally relevant to detection in microfluidic formats, as many microchip assays rely on fluorescence detection. Nevertheless, the dynamic or self-coating property of polysaccharides and derivatives is attractive for treating column surfaces.^{56,87}

3.4.1. Dextran. Hydrophilic polymer dextran is characterized by a low viscosity (6 cP at 6% concentration⁴²). As a result,

Fig. 3 Increasing the Mg^{2+} concentration in PVP solution increases the hydrodynamic radius of PVP (R_h) and decreases the screening length (ξ), thus improving the separation efficiency of the PVP sieving matrix. Reprinted with permission from ref. 39, © 2008 American Chemical Society.

the dextran is easily replaceable after each electrophoresis run with >300 runs demonstrated in a single CE column.¹³ Dextran is also UV-transparent so that on-line label-free protein detection is possible, again, perhaps not a strong advantage for microfluidics.^{13,100} The electrophoretic separation performance of dextran is similar to the popular LPA sieving matrix.⁴³

Baba's group used a dextran sieving matrix (from *Leuconostoc mesenteroides*) and MC non-covalent coating in a PMMA microdevice.⁴² To coat the microchannels, a MC solution was injected and dried before the electrophoresis runs. A set of molecular-mass standards ranging from 14.4 kDa to 29.0 kDa were well resolved in 6% dextran (5 mM Tris-HCl and 3.5 mM SDS). At higher dextran concentrations, separation resolution declined which was attributed to a reduced difference between EOF mobility and SDS-protein mobility.²⁷ Instability of non-covalent MC coating was an issue; the coating only worked for 1 run. The separation efficiency ranged from 4.4 to 5.6×10^5 plates per m.

Whey proteins (α -lactalbumin, β -lactoglobulin, and BSA) were size-separated in an SU-8 microfluidic chip for allergenic product analysis in food.⁴³ A dynamic surface coating of 10% EOTrol LN (*i.e.*, acrylamide derivative) was used for EOF and protein-adsorption suppression. After optimization, 10% dextran (425–575 kDa) in 3.5 mM SDS and 5 mM borate buffer was used as separation media. The three proteins were resolved in <300 s (detection point was 3 mm). The separation efficiency was 1.8×10^5 plates per m for α -lactalbumin and an order of magnitude smaller for β -lactoglobulin and BSA.

3.4.2. Pullulan. Pullulan is a natural polymer produced from starch by the fungus *Aureobasidium pullulans*. Pullulan was demonstrated in CE-format SDS protein electrophoresis assays.^{96,101} The low viscosity of pullulan was a chief selection criteria.^{101,102} UV absorbance was 1 order of magnitude lower than that of LPA (at 214 nm).⁹⁶ An 8% pullulan sieving matrix yielded similar separation performance to that obtained by slab-gel SDS-PAGE.⁴⁵

Griebel *et al.* reported a 2D gel capillary electrophoresis (2D-CGE) in a PMMA microfluidic chip.⁴⁶ The first dimension was isoelectric focusing (IEF) using an immobilized pH gradient (IPG). The second dimension was SDS CGE using a 15% pullulan in 0.1 M 2-(cyclohexylamino)-ethanesulfonic acid (CHES), 0.1 M Tris-HCl and 0.1% SDS buffer as a sieving matrix. The second dimension comprised an array of 300 microfabricated parallel capillaries. Pullulan was prepared by heating the precursor solution (50 °C) and vigorous stirring. For EOF suppression, the authors used a multilayer coating process consisting of: (1) copolymer of maleic acid anhydride and vinylmethylether with diallylamine, (2) allylglycidyl agarose, and (3) surfactant Surfynol 104. Proteins transferred to the second dimension were separated in 20 min with an excellent inter-capillary reproducibility.

3.4.3. Methylcellulose (MC). MC is relatively hydrophobic and, thus, often used for coating of polymeric microfluidic chips; MC does not adsorb well on glass surfaces.⁵⁶ A MC sieving matrix is usually used with a detergent for proper dynamic coating and separation resolution.⁴⁷ Interestingly, MC solution at less than the entanglement point (0.01%) can still separate

proteins as hydrogen bonding yields entanglement of sparsely located cellulose fibers.¹⁰³ Baba's group used a combination of MC and the nonionic detergent polysorbate (Tween 20) as self-coating reagent and sieving matrix for native protein separations in a PMMA chip.⁴⁷ TI (20.1 kDa), BSA (66.3 kDa), and amyloglucosidase (100 kDa) were separated in <100 s in a 20 mM Tris-HCl buffer containing 2% MC and 0.02% Tween 20. The separation efficiency was 3.6×10^5 plates per m for TI and an order of magnitude lower for the two other proteins. The addition of Tween 20 was observed to play an important role in preventing protein adsorption on the hydrophobic PMMA surface, thus supporting polymer entanglement (for protein separation)⁴⁷ and reducing the injection pressure required for pressure loading of a sieving matrix of concentrated MC solution (>1.5%) into microfluidic channels.

3.4.4. Hydroxypropylcellulose (HPC). HPC has been demonstrated as a sieving matrix in CE of proteins¹⁰⁰ and, recently, in microfluidic DNA⁹³ and protein separations.⁵⁰ HPC is a thermo-responsive polymer, with viscosity dependent on temperature. The gel form may be heated for easy loading (lowering viscosity) and cooled for electrophoresis (increasing viscosity).⁹³ HPC is often used as a dynamic-coating additive to prevent protein adsorption and to reduce EOF.^{86,104}

Wang *et al.* analyzed lipoproteins using gel electrophoresis in a PDMS/glass microfluidic chip.⁵⁰ HPC (80 kDa) and n-dodecyl- β -D-maltoside (DDM) in 50 mM MOPS (3-morpholinopropanesulfonic acid) buffer was used as a sieving matrix. Pre-coating the PDMS surface with a mild nonionic detergent (DDM) for 10 min before electrophoresis successfully suppressed EOF and protein adsorption. The optimal HPC concentration was 0.6%. At higher concentrations, protein migration was retarded and injection of the polymer solution was difficult due to viscosity. The lipoproteins HDL (high-density lipoprotein), sdLDL (small, dense low-density lipoprotein), and LDL (large buoyant LDL) were separated in <3 min with acceptable assay reproducibility. A separation efficiency of 1.5×10^6 plates per m was obtained for HDL.

3.4.5. Hydroxyethylcellulose (HEC). HEC acquires a stiff and extended conformation in solution, resulting in a robust entangled polymer network even at a low concentration.^{49,51} Separation resolution has been reported as comparable to that obtained with LPA.⁷⁵ The viscosity of HEC is relatively low (150 cP at 5%).⁵³ Disadvantages of this cellulose derivative include the need for purification and batch-to-batch variability in composition.⁷⁵ Although HEC was frequently used in DNA⁷⁵ and protein analyses in CE formats,¹⁰⁵ microfluidic CGE has seen limited use of the material.⁴⁸ HEC has been employed as a dynamic surface coating reagent in microfluidic isoelectric focusing.^{52,53,84}

Landers' group compared common sieving polymers including PEO (200 kDa), HPC (100 kDa), dextran (2000 kDa) and HEC (250 kDa) for separation of eight standard ladder proteins using microchip electrophoresis.⁴⁸ For dextran, additional covalent surface coating was required for EOF suppression. The viscosity of PEO made pressure injection into the microchannel difficult. The authors reported that HEC and PEO showed the best resolving power and background fluorescence

levels. The same group also used HEC to separate proteins in a microfluidic IEF assay.⁵² Microchannels were incubated with 2.5% HEC for 10 min for coating. Then 1% HEC in the carrier ampholyte solution (pH 3–10) was used to separate the naturally fluorescent proteins R-phycoerythrin (RPE), B-phycoerythrin (BPE), and green fluorescent protein (GFP).

Fan's group published a series of studies regarding IEF in COC chips using HEC dynamic surface coatings.^{53,84,85} COC is a hydrophobic plastic substrate and, thus, protein adsorption of focused proteins is of great concern.⁵³ In their first two papers, a mixture of 0.73% HEC (90 kDa) and 1.83% HPC was used as a sieving matrix.^{84,85} Later, they optimized the separation media so that lower levels of HEC (0.2%) were effective at reducing EOF and protein adsorption.⁵³ BSA and GFP isoforms were successfully separated in a solution of 2% carrier ampholytes (pH 3–10) and 0.2% HEC. A 0.2 pI point difference was baseline resolved in <3 min.

3.4.6. Hydroxypropylmethylcellulose (HPMC). Another cellulose derivative used for CGE of DNA (dsDNA) is HPMC.^{7,75,106,107} HPMC is also low in viscosity (50 cP for 2% in water¹⁰⁶) and easily replaceable for repetitive electrophoresis runs. HPMC is also frequently used to suppress analyte adsorption and EOF.^{57,81} Additives such as mannitol or glycerol are added to HPMC solution in order to enhance separation performance.⁵⁴

Lin *et al.*, reported microchip CGE coupled with ITP (isotachopheresis) for sample stacking.⁵⁵ A solution of 1% HPMC in $1 \times$ TBE buffer was used as a sieving matrix in a PMMA chip. Protein markers (TI, CA, alcohol dehydrogenase, BSA, β -galactosidase, myosin) were enriched 21–40 times by ITP before the electrophoresis separation.

Baba's group studied the dynamic surface coating properties of MC, HPMC, PVA, and PVP on PMMA microchannel surfaces and the influence on electrophoresis of non-denatured proteins.⁵⁶ These researchers found HPMC reduced protein-adsorption most effectively, among the four water-soluble polymers considered. HPMC was also observed to be effective at attenuating EOF. Although an HPMC concentration below the entanglement point (*e.g.*, 0.2%) was used in the background electrolyte, this amphiphilic polymer interacted with proteins, causing differential reduction of protein mobility. Such differential mobility change may have facilitated protein separation even in a dilute polymer solution. A separation efficiency of 8.2×10^5 plates per m was obtained for 0.2% HPMC. The same group used a similar PMMA microchip to analyze lipoproteins HDL, low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL), biomarkers important in assessing cardiovascular system health.⁵⁷ As lipoproteins strongly adsorb to the hydrophobic PMMA surface, a dynamic coating of HPMC (up to 0.5%) alone did not suppress protein surface adsorption to satisfaction. Thus, SDS was added to coat the protein and PMMA surface with negative charges, reducing lipoprotein adsorption. HPMC (>0.05%) effectively suppressed EOF. Depending on polymer concentration, the mode of separation was different; at low HPMC concentration, lipoproteins were separated by zone electrophoresis ($\sim$ 0.05%). At a higher HPMC concentration (1%), three lipoproteins were separated by molecular sieving.

3.5. Polyethylene oxide (PEO)

Polyether PEO has been extensively studied in DNA separations *via* CE due to favourable surface coating properties, simple channel reloading after HCl flush, and reduced EOF.^{54,75,108} In addition, a relatively low viscosity (1200 cP for 1.5% (ref. 99)) and low UV absorbance at 214 nm are attractive properties for PEO as a molecular sieving matrix.⁷ Consequently, PEO is a commercial sieving matrix for SDS CGE (*e.g.*, Beckman SDS 14-200).^{59,109} Unlike LPA, dextran, and other popular polymers, PEO is nonreactive with post-column fluorogenic labelling reagents like NDA (naphthalene-2,3-dicarbaldehyde).⁵⁹ The separation power of 3% PEO SDS CGE has been reported as comparable with the performance of slab-gel SDS PAGE.¹ On-column fluorescence labelling and CE separations using four different polymer solutions (PEO, HPC, LPA and dextran) indicated that PEO offered the highest resolving power, no need for additional coating process, negligible interaction with dye, and low viscosity (100 kDa PEO).⁴⁸ However, hydrolysis in alkaline media is a downside of this useful polymer.^{110,111} PEO is more hydrophobic than LPA, which may adversely impact protein separation efficiencies.¹⁰⁸ Degraded separation efficiency was observed at temperatures above 25 °C, potentially constraining the maximum electrical power applied.⁶⁰

Cooke *et al.* used a mixture of PEO and PEG (from the commercial SDS 14-200 kit, Beckman Instruments) to separate a protein molecular-mass ladder of 14-205 kDa as well as crude fetal serum, chicken egg white, and bovine milk in an SDS CGE format.¹⁰⁹ Impressive reproducibility over 400 runs was demonstrated. This high-performance sieving matrix was transferred to a microfluidic format by Schultz's group.⁶¹ A molecular mass marker ladder of 9–116 kDa was separated in an uncoated 4.5 cm glass microfluidic channel with time-based separation efficiency 20× higher than that of a commercial CE system ($2\text{--}4 \times 10^5$ plates per m separation efficiency). Shadpour and Soper employed the same commercial PEO-based sieving matrix for 2D electrophoresis in a PMMA microfluidic chip.⁶² Here the first dimension was SDS gel electrophoresis and the second dimension was micellar electrokinetic chromatography. As a surface coating, the chip was primed with 2% MHEC before electrophoresis and 0.05% MHEC was added to the background buffer to suppress EOF. Proteins of similar molecular mass were resolved using this 2D separation: (1) actin (43 kDa), OVA (45 kDa), protein A (45 kDa); (2) BSA (66 kDa), Helix pomatia lectin (70 kDa); and (3) concanavalin A (104 kDa), lectin peanut agglutinin (110 kDa). Separation efficiency of 1.6×10^6 plates per m was obtained in the first dimension separation.

DeVoe's group also employed a PMMA microfluidic device for 2D electrophoresis,⁶⁰ but with free-solution IEF performed in a horizontal microfluidic channel with focused proteins subsequently "sampled" into an array of orthogonal channels where the species are analysed by electrophoresis. The device utilized *in situ* polymerized polyacrylamide gel plugs that separate IEF and electrophoresis channels to prevent chemical and fluidic crosstalk. Proteins in an *E. coli* cell lysate were analysed with the second dimension channel containing 2% PEO in 0.1% SDS, 10 mM Tris–CHES background buffer. The

PMMA chip surface was coated with a dual layer of *in situ* polymerized LPA and 4% PVA to prevent protein adsorption.

3.6. Interpenetrating network (IPN)

As can be seen in Fig. 4, two polymers with different physico-chemical properties can be mixed in a crosslinked form to yield an interpenetrating polymer network or a non-crosslinked "quasi-IPN".^{76,112,113} For example, a high molecular-mass LPA of an excellent separation capacity yet unfavorable viscosity and PVP with excellent surface coating properties can be combined to yield a sieving matrix with desired properties: high separation efficiency, appreciable dynamic surface coating, and low viscosity for easy gel load and replacement.¹¹³ Simply mixing two incompatible polymers results in a microphase separation, leading to poor sieving properties (Fig. 4c).¹¹³ Without cross-linking two polymers, polymerizing a monomer with another polymer yields a quasi-IPN with much less phase separation (Fig. 4d).¹¹² For DNA separations in CE formats, various quasi-IPNs have been investigated including: LPA + PVP,¹¹⁴ PDMA + PVP,¹¹³ poly(*N*-acryloyl-2-amino-2-hydroxymethyl-1,3-propanediol) + PVP,¹¹² and LPA + PDMA.⁹⁴ In addition to reduced analyte adsorption and lowered viscosity, higher separation efficiency was observed as compared to homopolymers of a similar molecular mass, after carefully tuning molecular mass and ratio of two incompatible polymers.¹¹⁴

While IPNs have been primarily applied to DNA separations, Song *et al.* demonstrated electrophoretic separations of basic proteins (cytochrome c, lysozyme and ribonuclease A) using a quasi-IPN of LPA and PDMA in a CE format.⁹⁴ Combining the

Fig. 4 Schematic of polymer networks formed by (a) PVP, (b) PDMA, (c) their simple mixture, and (d) interpenetrating network (IPN) of PVP and PDMA. Homopolymer (a) and (b) have a coarse network, not suitable high-resolution separations. A simple mixture (c) results in a microphase separation, and interfaces between the two phases do not have a sieving capability. (d) The IPN dramatically increases entanglement, yielding a stabilized small-pore-size network suitable for high-resolution biopolymer separation. Reprinted with permission from ref. 113, © 2002 Wiley InterScience.

high sieving capacity of LPA and the dynamic surface coating properties of PDMA, separation efficiencies ranged from 3.02 to 4.29×10^5 plates per m. The separation efficiency of the IPN containing a low molecular mass LPA (<1 MDa) was higher than that of a homopolymer LPA of much higher molecular mass (>3 MDa). The IPN matrix was loaded with ease (20 psi for 10 min) and EOF was well suppressed compared to a bare fused silica capillary. IPN appears suitable to adaptation to microchip protein electrophoresis.

4. Crosslinked polymers as separation matrices

Regardless of many aforementioned advantages of the replaceable polymer solutions, pressure-injection of linear polymer into narrow columns is difficult, because of viscosity limits.¹¹⁵ Owing to higher fluidic resistances, the loading challenge is even more pronounced in micro-to-nanofluidic devices with complex geometries. The low viscosity of the polymer precursor solution (monomer and porogen) affords easy channel filling with the desired polymer gel subsequently formed *via* chemical- or photo-initiation.¹¹⁶ Fabrication of complex gel structures⁶³ or even regionally patterned micro-chambers^{20–22} is feasible with crosslinked polymers. The pore size and density of the crosslinked monolith can be readily controlled in each specific zone designated for sample pre-concentration, separation, or immobilization.¹¹⁷ Moreover, stiffer networks appear to enhance separations,^{51,75} but are difficult to achieve with linear polymers as viscosity increases with stiffness. A major drawback of crosslinked polymer monoliths is difficulty in removing the material from micro-channels, which makes it difficult to recycle chips. Piranha etching is generally used to chemically decompose the polymer matrix in glass or borosilicate chips.¹⁷ The heterogeneity of the pore structure under certain conditions can pose potential problems, as only the average pore size of a monolith can be well-controlled.¹¹⁸

4.1. Polyacrylamide

Polyacrylamide (PA) was used for separation of serum albumins in the 1960s,¹¹⁹ and has become ubiquitous as a separation media for analysis of proteins. Two formats are most widely used: PAGE and SDS-PAGE. With applicability to a diverse array of measurement challenges and a long history, PA-based separation media have been applied to numerous formats. The miniaturization of bench-top PAGE,⁶³ SDS-PAGE,^{21,23} IEF,^{24,60} and immunoblotting^{17,64,117,120} have all been reported using conventional crosslinked PA. Adaptation of PA to microfluidic devices preserves the separation mechanism of slab-gel systems that have been extensively investigated. Thus miniaturization of PAGE-based separations benefits from the deep existing understanding of separation performance.

Hughes *et al.* developed an integrated microfluidic system for western blotting following SDS-PAGE²³ and IEF.²⁴ The researchers integrated a discontinuous PA gel supporting transient ITP and subsequent SDS-PAGE by photopatterning the

crosslinked polymer in a straight microchannel (Fig. 5). Benzophenone-functionalized methacrylamide co-monomer was incorporated into the polyacrylamide gels, thus allowing covalent UV-initiated immobilization of separated proteins. After brief UV exposure, the immobilized proteins were probed by antibodies, comprising a western blotting assay. The microfluidic approach overcomes several limitations of slab-gel system by greatly reducing antibody consumption (from $1 \mu\text{g}$ to 1 ng), human intervention and assay time (from days to hours), owing to miniaturization and automation of the otherwise laborious workflow.

A two-dimensional microfluidic system is reported for protein separations combining IEF and SDS-PAGE employing *in situ* photopolymerized PA gels.^{60,63} In an alternate approach to the microchannel array used in that work, a microchamber supported 2-D protein separations (Fig. 6).²² Tentori *et al.* formed spatially distinct PA gels by photopatterning; one for IEF

Fig. 5 Design and operation of the microfluidic western blot. (a) A glass microfluidic device with microchannels linking two fluid reservoirs (dye added for clarity). (Scale bar: 2 mm.) (b) The 80 min five-stage immunoprobe assay is completed in a single microchannel. (c) Schematic of microchannel cross-section depicting principle of the protein immobilization: analytes are electrophoresed through the reactive nanoporous hydrogel, exposed to UV, and covalently immobilized. (d) Schematic of reaction between polypeptide backbone and benzophenone groups. Ph denotes phenyl group. (e, left) SDS-PAGE of fluorescently labeled six protein ladder (black), complete in 60 s ($4\times$ magnification; band weights are 155, 98, 63, 40, 32, and 21 kDa). (e, right) Multiplexed immunoblot readout (red) in 40 min total assay times using primary antibodies for (i) OVA (ovalbumin), and (ii) β -gal (β -galactosidase), OVA, and TI (trypsin inhibitor); all at $1 \mu\text{M}$. Fluorescence micrographs is shown for red fluorescent primary antibodies (Ab^*). Reprinted with permission from ref. 23 and 24, © 2012 National Academy of Science.

with immobilized pH gradient and another for sizing of focused proteins.

The high mechanical strength and stiffness of crosslinked PA, which linear polymers lack, enabled development of free-standing PA gels for microfluidic electrophoresis.⁶⁵ This system does eliminate wall interactions and facilitates post-separation protein manipulation steps including protein staining, immunoblotting, and protein extraction – functions which can be troublesome to concatenate with enclosed channels. Concurrent PAGE in an array of 96 free-standing gel lanes was demonstrated.

Cyclodextrin–acrylamide gels have been reported to endure high electric fields (up to 700 V cm^{-1}) without bubble formation unlike conventional PA gels.^{115,121} The addition of cyclodextrin actually improved the lifetime of the monolithic gel column with resolution maintained for up to 1 month. Crosslinked polymers which can support high electric-field strength, heat, or mechanical strain may be desirable for both nanoscale and high-throughput devices.

PA is suitable for passivating microchip surfaces for protein separations. For nanoscale channels where the surface area to channel volume ratio becomes larger, surface treatment gains in importance for proteomic applications.¹²² Moreover, the versatile chemistry of acrylamide supports diverse functionalizations. Interested readers should consult the reviews published on this topic for photopatterning¹²³ and immobilization.⁷⁹

4.2. Acrylate/methacrylate

Mask-based photopolymerization of acrylate- or methacrylate-based monoliths allows formation of sophisticated and integrated structures in specific areas of a microchip. The photo-initiation of benzophenone¹²⁴ is also employed to integrate various functionalities including surface modification, pre-concentration and separation in a single channel.¹¹⁶ Creation of stable acrylate- or methacrylate-based monoliths by forming

covalent bonds between the monolith and channel walls in polymer devices has been reported.¹²⁵ Integration of solid-phase extraction (SPE) and HPLC in a COC microchip was accomplished using *in situ* photopolymerized polymethacrylate monoliths.⁶⁷ The integrated chip design includes a 15 cm long separation column for HPLC coupled to a 5 mm-long methacrylate trap column, which functions as the SPE element. Removal of free dye and enrichment of fluorescein-labelled proteins was demonstrated to improve analytical sensitivity by $150\times$ and decrease peak width by $10\times$, after a microchip gradient LC separation.

Polymethacrylate monoliths support simultaneous pressure-driven and electrokinetic analysis in a single device. This capability allowed integration of pressure-driven reverse-phase liquid chromatography (RPLC) with IEF.¹²⁶ Sample proteins were concentrated and enriched by IEF prior to RPLC.

PEG-modified acrylate-based conductive hydrogels (PEG-diacrylate based) also support the simultaneous hydrodynamic and electrophoretic control needed for micro electric field gradient focusing (μEFGF), which concentrates biomacromolecules by careful control of electrokinetic and hydrodynamic flow (Fig. 7).¹²⁷ Tandem μEFGF was comprised of a lower conductivity hydrogel for analyte preconcentration and higher conductivity hydrogel for analyte separation. Performance was improved by use of phosphate buffer and high-mobility ions.⁶⁶ PEG-modified acrylate was also used to fabricate protein-resistant monolith for analysing the affinity between proteins and aptamers.¹²⁸

Liu *et al.* utilized a nanoporous methacrylate monolith within a microfluidic channel to trap and concentrate silver nanoparticles in a 3D matrix.¹²⁹ The SERS-active monolith was

Fig. 6 Design and operation of gel photopatterning for microchamber 2D electrophoresis (2DE). (a) Etched glass microdevice features a $3 \times 3 \text{ mm}^2$ microchamber flanked by channels for sample loading and electric field control. (b) The device houses contiguous PA gel regions with distinct chemophysical properties to define the IEF and PAGE separation axes. (c) Operation procedure of micro-2DE. Reprinted with permission from ref. 22, © 2013 American Society of Chemistry.

Fig. 7 (a) Schematic of the μEFGF device. (b) Separation of three proteins in a $120 \mu\text{m}$ I.D. EFGF channel. The counter flow rate was 10 nL min^{-1} and the applied voltage was 800 V . Peak identities: (1) FITC- β -lactoglobulin A, (2) RPE, and (3) GFP. Reprinted with permission from ref. 66, © 2009 Elsevier.

capable of label-free detection of proteins. Modified polymethacrylate can present hydrophobic to hydrophilic properties. An advanced 2D thin layer chromatography assay harnessed hydrophilic microchannels seated on a planar layer of superhydrophobic monoliths.¹³⁰ The superhydrophobic 50 μm -thick monolith layer was composed of poly(butyl methacrylate-*co*-ethylene dimethacrylate), while the hydrophilic channel was formed by photopolymerization of a mixture containing 2-acrylamido-2-methyl-1-propanesulfonic acid, 2-hydroxyethyl methacrylate, and benzophenone. Proof-of-concept peptide separations were demonstrated.

5. Other noteworthy materials

5.1. Stimuli-responsive polymers (block copolymer gels)

Block copolymers are emerging materials for separation media with versatile and adjustable properties including pore size, hydrophilicity, and stimuli-response. Designing 'smart' microfluidic assays with stimuli-responsive polymers is an area of increasing interest as use of multi-functional polymers allows properties can be controlled or adjusted by the external chemical and physical stimulus, such as temperature,¹³¹ pH,¹³² electric field,¹³³ light,¹³⁴ or ionic strength.¹³⁵ For example, thermally-responsive (thermo-responsive) polymers have been extensively studied for DNA analysis in CGE formats because their viscosity can be tuned by temperature.^{76,93,136} While concentrated high-molecular-mass polymer such as LPA can provide excellent separation efficiency, pressure as high as 1000 psi is required to fill a capillary with the polymer solution.⁹³ Noting that glass or plastic microfluidic chips can sometimes withstand just moderate pressures (<200 psi,¹³⁶ with the exception of solvent-bonded PMMA⁸⁶), "viscosity switching" is an attractive property of a sieving matrix. Reversible viscosity switching supports gel replacement for the subsequent electrophoresis runs at the low-viscosity state, and uncompromised separation performance at the high-viscosity state (Fig. 8).¹³⁶

Various polymers including HPC,^{93,137} PNIPAAm [poly(*N*-isopropylacrylamide)], its copolymer P(NIPAAm-*acrylamide*-Bis *acrylamide*),¹³⁸ and its grafted polymer LPA-*g*-PNIPAAm¹³⁶ and PNIPAAm-*g*-PEO,⁷⁶ PDEA [poly(*N,N*-diethylacrylamide)]⁹³ and its copolymer PDEA-*co*-PDMA, PPO [poly(propylene oxide)] and its popular triblock copolymer Pluronic [(PEO)_{*x*}(PPO)_{*y*}(PEO)_{*x*}]⁷⁶ and grafted polymer LPA-*g*-PPO¹³⁶ have been studied for separation applications. Among these polymers, a handful are applied to protein separation in CE formats^{68,139} while many of the stimuli-responsive polymers have been actively applied for actuators, micropumps, and microvalves.¹⁴⁰ The effect of PNIPAAm microstructure on the resolution of DNA sequencing has been reported.¹⁴¹ The copolymer P(NIPAAm-*acrylamide*-Bis *acrylamide*) was used for protein separation in CGE format by Wang *et al.*¹³⁸ The copolymer was in a clear low viscosity state at room temperature (22 °C), switching to a turbid high-viscosity state at 33 °C. The authors used Joule heating generated by the electrophoresis current to increase the polymer viscosity. Protein standards ranging from 14.4 kDa to 97.4 kDa were separated. The copolymer also exhibited a surface-coating property. Many of thermo-responsive polymers have hydrophilic backbones

Fig. 8 Schematics representing reversible association of LPA-*g*-PPO [poly(propylene oxide)] depending on temperature (above or below lower critical solution temperature or LCST). Thermal association of PPO side chains are initiated and the entanglement of polymers is increased. Reprinted with permission from ref. 136, © 2009 Wiley InterScience.

(*e.g.*, LPA) and grafted or copolymerized hydrophobic chains (*e.g.*, Bis-*acrylamide*, PNIPAAm).^{68,136} Hence, hydrophobic interactions with proteins could influence protein separation performance.⁶⁸

(PEO)_{*x*}(PPO)_{*y*}(PEO)_{*x*} is an amphiphilic triblock copolymer containing PEO as a hydrophilic block and PPO as a hydrophobic block. PEO-PPO-PEO triblock copolymers have potential advantages as an alternative separation medium due to an enhanced dynamic coating¹⁴² and thermo-reversible character, which are useful for loading polymer solution into microchannels at low temperature (*e.g.*, Pluronics).⁵⁸ The Jung Lab showed that the separation resolution afforded by a triblock copolymer solution was improved compared to that attained in conventional PDMA.¹⁴³ The triblock copolymer solution was successfully employed for analysis of pathogens¹⁴⁴ and multiplexed ligation-dependent probe amplification.¹⁴⁵ Acrylamide-based triblock copolymers including polydimethylacrylamide-*block*-polyethylene oxide-*block*-polydimethylacrylamide (PDMA-*b*-PEO-*b*-PDMA) allowed efficient EOF suppression for capillary zone electrophoresis and supported appreciable separation efficiency improvements over PEO-*b*-PDMA diblock copolymers.¹⁴⁶

Thermo-responsive pseudogels composed of phospholipids were utilized for hydrodynamic delivery of sample and sieving matrix. The phospholipid mixture becomes gel-like at physiological temperature, and its viscosity can be thermally controlled from 24 °C (low viscosity, suitable to introduction into capillary) to 29 °C (high viscosity, works as a sieving matrix).¹⁴⁷ The separation of oligosaccharide¹⁴⁸ and DNA¹⁴⁹ has been demonstrated with excellent separation performance.

The molecular sieving characteristics of a pH-responsive diblock copolymer, polystyrene-*b*-poly(4-vinylpyridine) (PS-*b*-P4VP) was highlighted in diffusion experiments using isoporous membranes with pore sizes of 34 nm.⁶⁹ BSA and globulin- γ (IgG) were effectively resolved in the system. Furthermore the membrane allowed the selective separation of similarly sized proteins based on charge effects. After quaternization of the membrane, proteins were fractionated based on isoelectric point by varying the pH.

5.2. Carbon nanotube (CNT) and CNT-polymer composites

Single-walled carbon nanotube (SWCNT) and multi-walled carbon nanotube (MWCNT) have seen applications in DNA^{95,150}

and protein separations.¹⁵¹ CNTs form a “pseudostationary phase” in a background buffer in a manner similar to behaviour of a polymer solution above the entanglement concentration.¹⁵² The pseudostationary phase acts as a molecular sieve during electrophoresis. CNTs are a hydrophobic material and thus often functionalized (*e.g.*, hydroxyl, carboxyl and carbonyl groups) prior to aqueous suspension. Such surface-functionalization imparts a negative charge,¹⁵² making charge interactions with analytes and/or microchannel surfaces important.¹⁵⁰ The black colour of CNTs may interfere with UV or fluorescence detection. To address this, Xu and Li used a non-optical detection technique C4D (capacitively-coupled contactless conductivity detection) for CGE of DNA. These researchers found that adding MWCNT to 1% PVP sieving matrix improved the limit of detection, compared to a 1% PVP solution.¹⁵⁰ Zhou *et al.* employed a double network consisting both of PDMA-functionalized MWCNTs and LPA-PDMA quasi-IPN for DNA separations.⁹⁵ Their rationale for using PDMA-functionalized MWCNT was insufficient solubility and biocompatibility of carboxyl-functionalized CNTs. The authors speculated that combination of flexible LPA-PDMA IPN and rigid PDMA-MWCNT would improve the stability of the molecular-sieving network (Fig. 9). Analyte adsorption was reduced as PDMA-MWCNTs have an excellent coating ability. The authors also reported that the viscosity of the double network was low and sieving performance was excellent by combining a low molecular-mass LPA (3.3 MDa) and a rigid network of PDMA-MWCNT.

A handful of studies have explored using a CNT-modified crosslinked sieving matrix for protein separations.^{151,153,154} In one study, SDS-PAGE of a protein standard was demonstrated using a CNT-modified polyacrylamide slab gel.¹⁵³ In a related study, a native PAGE separation of apolipoprotein and complement C3 protein was performed in the same slab-gel format.¹⁵⁴ Photopatterned CNT-hydrogel composites have also been utilized for microchip electrophoresis of casein, BSA, and IgG.¹⁵¹ Here, SWCNTs were first functionalized with PEG-acrylate (SWCNT-PEG-acrylate) and then photopolymerized with acrylamide monomer, crosslinker EDMA (ethylene-glycol dimethacrylate) and photo initiator DPA (α,α -dimethoxy- α -phenylacetophenone) inside a glass microfluidic chip (Fig. 10a). SWCNTs were integrated within the polymer to reinforce the polymer network to withstand hydrodynamic flow or EOF (Fig. 10b and c). Protein adsorption and EOF were reduced by

the CNT-composite gel. Intriguingly, the required separation distance was shorter than that for a PEO polymer-solution sieving matrix.

5.3. Free-solution electrophoresis

Although free-solution electrophoresis does not inherently employ a polymer sieving matrix and is, thus, beyond the scope of this review, these matrix-free approaches may be of interest to highly-integrated nanofluidic devices. In particular, the heterogeneity of the polymer matrix is more pronounced and results in irreproducible separations when the length scale of the polymer pores are on the order of those of the channel diameter. In such cases, free solution electrophoresis offers an alternative option. The option is of particular interest for proteins prone to non-specific adsorption, though intensive surface treatment considerations, exaggerated by the high surface-to-volume ratio of nanochannels, are required. Excellent reviews of these topics can be found in ref. 155 and 156.

End-labeled free-solution electrophoresis (ELFSE) or drag-tag electrophoresis^{157,158} alters the apparent electrophoretic mobility of a tagged analyte by the number of electric charges added (proportional to the molecular weight of the analyte). A macromolecule attached to the ends of a DNA or polypeptide chain provides friction to amplify mobility differences. The friction-inducing macromolecules, often called ‘drag tag’, should have low net electric charge and provide substantial yet uniform friction. For this purpose, various engineered peptide drag tags were developed; monodisperse synthetic peptoid drag tags,^{159,160} recombinant proteins,¹⁶¹ even longer and monodisperse engineered polypeptides,^{162,163} and end-attached micelles.¹⁶⁴ The principle of the ELFSE is fundamentally different to matrix-based separation methods, with different physical limitations. ELFSE has the potential to resolve short DNA fragments (*e.g.* <1000 bases) in high electric field, where the resolution in gel electrophoresis readily declines, particularly in high electric fields (*e.g.*, reptation with stretching).¹⁶⁵ The polymer matrix and drag-tag methods complement each other, and a potential hybrid separation system has been developed to accelerate Sanger sequencing.¹⁶⁶

5.4. Ionic liquid

Functionalized ionic liquids are an emerging new material for both surface treatment¹⁶⁷ and separation medium functions.¹⁶⁸ Dynamic coating with imidazolium-based ionic liquids provides efficient suppression of basic protein adsorption,¹⁶⁹ while 1-butyl-3-methylimidazolium tetrafluoroborate has proven to be effective for both acidic and basic proteins.¹⁷⁰

A hydrophilic ionic liquid, 1-butyl-3-methylimidazolium dodecane sulfonate (BAS), has been successfully adopted for microfluidic protein separation.¹⁶⁸ This multi-functional ionic liquid performed multiple roles as a surface modifier, a supporting electrolyte and a sieving matrix. The exploitation of BAS in microfluidic systems not only offers a guaranteed ionic strength to enhance EOF, but also effectively eliminates protein adsorption the surface of the PDMS microchannels.

Fig. 9 (a) Schematic of the formation of quasi-IPN (PDMA-LPA)/MWNT-PDMA double-network composite sieving matrix. (b) TEM images of MWNT-PDMA in quasi-IPN solution of PDMA-LPA. Reprinted with permission from ref. 95, © 2008 Wiley InterScience.

Fig. 10 (a) Chemistry of the CNT-reinforced hydrogel. (b) The hydrogel without integrated CNTs was damaged after flushing while (c) the CNT-hydrogel composite was not damaged after flushing. Reprinted with permission from ref. 151, © 2008 Wiley InterScience.

6. Concluding remarks

Analysis of complex proteinaceous samples benefits tremendously from electrophoretic separations. Implemented in slab-gels and capillaries, protein electrophoresis in microdevices is now also a cornerstone of lab-on-a-chip applications. To obtain the desired high separation performance in columns with small dimensions (*i.e.*, capillaries, microchannels), polymeric materials are favored as molecular sieves. Tuning the sieving capacity of a polymer to match the needs of the separation and the application is an advantage of said materials.

Crosslinked and non-crosslinked gels, traditional polymer materials, have been successfully adapted to the microfluidic formats. Commercial non-crosslinked gels (polymer solutions) based on PEO (Beckman) and PDMA (Agilent) are the good examples of microfluidic adaptation. Because of easy preparation, long shelf life, and easily tunable sieving properties, we envision continuous use of non-crosslinked polymer solutions in microfluidic protein analysis. One of the strongest benefits of using the polymer solutions is that the solution can be replaced if deteriorated. Although theoretically sound, there are technical challenges in gel loading because injecting the solutions to a microfluidic chip without leakage by applying positive pressure (*i.e.*, fitting problem)¹⁰⁸ is not straightforward, especially so when a microfluidic network has a high fluidic resistance (*i.e.*, complex, long and slender channels). Thus, new low-viscosity polymers possessing high molecular-sieving power will be sought continuously. Polymers not adapted in microfluidic format yet, but used in CE format include polysaccharides derivatives (cationic starch derivatives,^{88,171} amylose, carboxymethyl amylose sodium salt, laminaran,⁷⁶ glucomannan, TreviSol,⁵⁴ galactomannans¹⁷²), PVA,⁷³ PEG with fluorocarbon tails, and linear polyAAP (poly-*N*-acryloylaminopropanol).^{40,108} These polymers could be employed for microfluidic protein separation. In order to improve separation performance beyond that of a homopolymer, various combinations of polymers *i.e.*,

grafted polymer, copolymer, and interpenetrating network will likely be studied further. Especially thermo-responsive polymers caught our interest because one could exploit otherwise detrimental Joule heating by electrophoresis current to control viscosity, possibly eliminating external heating elements.⁶⁸ Crosslinked polymer gels have also been successfully adapted in microfluidic protein electrophoresis and complemented the disadvantages of the polymer solutions. Crosslinked gels can be formed in complex and narrow channels with no problem. Moreover, the polymer density and pore size can be easily manipulated by adjusting composition of precursor mixture. Among several crosslinked polymers attempted in microfluidic protein analysis, PA currently prevails because direct miniaturization of the familiar slab-gel PAGE is possible without any complication like the viscosity problem of LPA. However, the development of alternative material is desired in the case of nanoscale device that higher resolution and highly uniform pore distribution is required.

When selecting new polymers caution should be made to avoid hydrophobic interactions with proteins which would degrade separation performance. When using polymer solution, one should always consider wall coating to suppress EOF and analyte adsorption. With ever increasing use of polymer substrates, surface passivation seems more and more critical. Unless the polymer has self-coating property, dynamic-coating additives need to be included in a sieving matrix. There have been numerous covalent surface coating approaches^{79,81,83,91} but the dynamic coating is, in principle, much simpler to employ in microfluidic format. The additives including EPDPA (epoxy PDMA), poly DADMAC [poly(diallyldimethylammonium chloride)], PSS (polystyrene sulfonate), PAH [poly(allylamine hydrochloride)]⁹¹ could be used for dynamic surface coating in protein electrophoresis.

We foresee expanding use of nanomaterial (*e.g.*, CNT, nanoparticles)-composite crosslinked polymers in protein separation.⁷⁶ The CNT-enforced gel is interesting in that

hydrodynamic-electrokinetic hybrid fluidic control is possible owing to high mechanical strength. This may enable multi-step microfluidic protein assay such as western or eastern blotting because blotting, staining and destaining reagents could be loaded quickly by hydrodynamic injection over the gel where proteins were separated. One downside is the black colour of CNT, thus transparent polymer nanowires¹⁷³ could replace the CNT for optical protein detection. Beyond the said advantage, photopatterning of crosslinked polymers are attractive because it allows integration of multiple assay functionality in a single device. However, bubble formation by gel breakage/shrinkage, especially gel of a low monomer concentration, is problematic. Thus one could replace the dilute crosslinked gel (low %T) with polymer solution and use it in conjunction with the high-monomer-content gels (high %T) to render the multi-step protein assay more robust. As such, by combining benefits of crosslinked gel and non-crosslinked gel, gel-electrophoresis-based microfluidic protein assays could be improved.

Microsystems not only augment conventional separations, but for multiplexing and integrated assays, such systems open up new possibilities. In the next decades, the advances of separation media will be required in reproducibility, separation resolution, detection sensitivity and ease of fabrication, together with the integration of different functional operations.

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