Functional materials for microscale genomic and proteomic analyses

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The design of functional materials for genomic and proteomic analyses in microscale systems has begun to mature, from materials designed for capillary-based electrophoresis systems to those tailored for microfluidic-based or 'chip-based' platforms. In particular, recent research has focused on evaluating different polymer chemistries for microchannel surface passivation and improved DNA separation matrix performance. Additionally, novel bioconjugate materials designed specifically for electrophoretic separations in microscale channels are facilitating new separation modalities.

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Abbreviations

DEA	*N,N*-diethylacrylamide
DMA	*N,N*-dimethylacrylamide
dsDNA	double-stranded DNA
epoxy-(AAG-AA)	poly(acrylamide-*co*-allylamide of D-gluconic acid-*co*-allylglycidyl ether)
EOF	electro-osmotic flow
LPA	linear polyacrylamide
NIPA	*N*-isopropylacryamide
PVP	poly(vinyl pyrrolidone)
ssDNA	single-stranded DNA

Introduction

The miniaturization of chemical and biological analysis systems (which are often called 'micrototal analysis systems (μTAS)' or 'laboratories on a chip') enables vast improvements in their performance both in terms of the speed of a typical analysis and in throughput, in comparison with more traditional 'full-size' instrumentation [1]. Perhaps the first and most striking example of these improvements is provided by the great success of capillary electrophoresis in speeding up the sequencing of the first human genome [2]. This accomplishment would have required many more years with traditional full-size slab-gel technology. The miniaturization of DNA electrophoresis from slab-gels to capillary arrays only represents the first step in a biotechnological revolution. This revolution will be driven by ever-greater miniaturization of biochemical analysis platforms, in particular onto integrated microfluidic devices or chips made from glass or plastic [3,4]. In electrophoresis-based systems, the rapid speed of separation obtained on chips stems primarily from efficient injection strategies and, hence, the much shorter distances that molecules must travel to be sufficiently separated. Already, microfluidic systems show great potential for increasing the speed of data-collection from many types of biological samples, as evidenced by the successful commercialization of the first robust microscale analysis systems [3,5]. Systems of interest can be divided into two major classes: those that use electrophoresis as their primary biomolecule separation/information-gathering modality, and those that function on the basis of other physical mechanisms (such as DNA hybridization) [5,6]. This review will focus on functional materials for the former of these two classes of systems — those based on electrophoresis. Primary analytes of interest include both DNA and proteins; the development of materials for genomic analysis is more advanced, and will be emphasized to a greater degree in this review.

Classes of functional materials for microscale bioseparations

Numerous reviews on the fabrication and operation of electrophoresis-based microscale systems exist [7–13]. In this review, however, we will focus on the consumable materials that allow these systems to function. The materials that will be addressed can be broken into three functional classes: separation materials, wall coatings, and novel bioconjugate materials.

Materials for optimal biomolecule separation performance

The first, and perhaps most important, class of materials is the bulk medium in which biomolecule separation occurs. Typically, this medium must be loaded into a microchannel before use and unloaded afterwards. This can be an aqueous buffer solution alone or a polymer solution dissolved in a buffer, and might also include a gradient in pH, concentration or electric conductivity along the separation axis.

In response to the human genome project, which required high-throughput DNA separation technologies for both restriction mapping of double-stranded DNA (dsDNA) and for separating the single-stranded DNA (ssDNA) fragments generated by the Sanger method of DNA sequencing, the development of novel separation materials for DNA is receiving much attention. Recently, Albarghouthi and Barron [14] reviewed the various polymeric materials that have been used as entangled separation matrices for DNA separations. The electrophoresis community largely agrees that highly entangled solutions of linear polyacrylamide (LPA) have generally provided the best sequencing separations, making it possible to separate DNA molecules differing by a single base for fragments up to 1300 bases in length [15]. Numerous researchers have investigated other polymers, but none has yet improved upon this performance.

Poly(vinyl pyrrolidone) (PVP) was recently tested as a DNA sequencing matrix and was shown to provide the separation of just over 300 contiguous DNA bases in sequencing applications [16]. The limiting characteristic of this matrix is excessive nonspecific (hydrophobic) interaction

between the sieving matrix and the fluorescent dyes, which obscures the separation of larger DNA molecules. Hydrophilicity of a polymer matrix was shown recently to be a critical determinant of attainable read length [17]. However, the relative hydrophobicity of PVP has been shown to give increased resolution in the separation of modified DNA oligomers, which are used in genotyping assays. Cohen and coworkers [18] showed that this increase in resolution results from a combination of electrophoretic and chromatographic separation mechanisms. Finally, Landers and coworkers [19] have shown that PVP can be used as an effective surface coating for microscale polymerase chain reactions (PCR) in chips, and as a surface coating and sieving matrix for neat PCR products [20] and heteroduplex genotyping analysis [21].

Although LPA provides superb DNA separations for long-read sequencing, it requires the use of a stable microchannel wall coating for suppression of electro-osmotic flow and prevention of analyte adsorption (discussed below). Some polymers do not require these procedures, because they adsorb to the wall spontaneously to form a coating, and effort has been devoted to developing these types of polymers as high-performance sequencing matrices. Song *et al.* [22] recently investigated the use of various novel polymers, including copolymers and polymer blends, that obviate the coating procedures required for separations within an LPA matrix. A homopolymer of *N,N*-dimethyl-acrylamide (polyDMA) was also investigated as a 'self-coating' matrix for DNA sequencing. Using relatively high molecular mass polyDMA (5.2 MDa) under optimized conditions, separation of DNA up to ~1000 bases was achieved, although the accuracy of base-calling (a critical metric of performance) was not reported [22].

Random copolymers of acrylamide and DMA of various acrylamide:DMA ratios were investigated. Under optimized conditions with a comonomer ratio of 3:1 and a molecular weight ≈ 2.2 MDa, separations of up to 700 contiguous bases were achieved, although again with no specification of accuracy in base calling [23]. A polyoxybutylene-polyoxyethylene-polyoxybutylene triblock copolymer (BEB), which self-organizes by hydrophobic association, allowed for separation of dsDNA restriction maps with good performance. BEB will probably not perform well in more demanding ssDNA sequencing separations, however, because of its hydrophobicity [24]. The synthesis of copolymers can be difficult to control and reproduce, especially in the case of block copolymers, thus mixtures of LPA and polyDMA have also been investigated, but LPA:polyDMA polymer mixtures of many different ratios are immiscible. With ratios carefully chosen to avoid these incompatibilities, DNA sequencing in these aqueous polymer blends could be achieved with an impressive separation efficiency of 10×10^6 plates/meter and a nominal read-length of 730 bases (again without report of base-calling accuracy) [25].

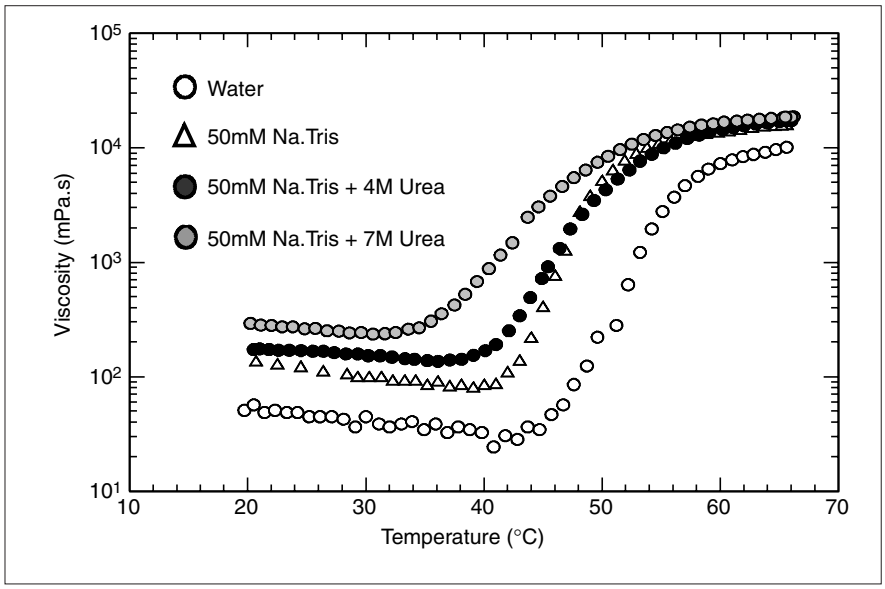
Taking a novel approach to matrix formulation, Liang *et al.* [26••] showed that the addition of a very small amount of montmorillinite clay into a polyDMA polymer solution resulted in an increase in DNA separation performance without an increase in analysis time. (Normally, an increase in one of these parameters comes at the cost of the other.) The authors postulate that this increase in performance was due to the clay serving as a dynamic cross-linking agent between separate polymer molecules, providing stabilization of the polymeric network.

The excellent performance of LPA is believed to result in part from its hydrophilic chemistry [17]. In an effort to create novel copolymers of high hydrophilicity, a sugar-bearing acryl monomer compatible with LPA-type polymers was synthesized and investigated for sieving performance in the absence of an additional capillary coating. The resulting copolymers of acrylamide and sugar-like β-D-glucopyranoside produced good separation of dsDNA ladders in the size range of typical restriction maps; the performance of this matrix for DNA sequencing was not reported [27•].

One of the greatest challenges in the implementation of polymeric solutions in microfluidic devices is the introduction of these separation matrices, which are usually quite viscous, into narrow channels. It is also desirable to force 'used' matrix out of the channel after each use and refill the channel with fresh matrix. This is especially true in the case of glass chips, which are relatively expensive to fabricate. Glass capillaries can be used in this manner for hundreds of analyses. Pumping these viscous fluids into the separation microchannel requires either very high pressures (>500 psi) or long times under lower pressures. The former strategy is incompatible with microfluidic devices owing to their relative mechanical fragility, and the latter is incompatible with fast, high-throughout separations. One ingenious method to address this challenge, investigated over the past few years, is the use of polymeric materials that exhibit two viscosity states or materials with a temperature-controlled 'viscosity switch'. This allows matrix loading in a low-viscosity state and electrophoretic DNA separation in a high-viscosity state. Viovy and coworkers [28••] have shown that matrices based on a hydrophilic polymer backbone of LPA, with short grafts of low molar mass *N*-isopropylacrylamide (NIPA) along the backbone, exist as low-viscosity polymer solutions below 40°C. Above this temperature the matrix viscosity increases by approximately two orders of magnitude, as shown in Figure 1. The transition is driven by hydrophobic association of the NIPA grafts above NIPA's phase-separation temperature of 32°C, as shown schematically in Figure 2. Thus, the polymer solution may be loaded into a microfluidic device at a low temperature (with a viscosity ~1000 centipoises [cP]), while at a high temperature the polymer serves as a good DNA sequencing matrix (viscosity ~10 000 cP) providing reads by capillary electrophoresis of about 800 bases (no base-calling accuracy given) [28••]. The only weakness of this study was that a necessary control, an experiment to show that the presence of the NIPA grafts and the subsequent hydrophobic association were necessary for good sequencing performance, was not done. Linear polyacrylamide is an excellent medium for

Figure 1

Plot of viscosity versus temperature demonstrating the thermogelling properties of hydrophobically modified polymers that exist as a low viscosity solution at low temperature, and a high viscosity solution at elevated temperatures. The viscosity was measured for the same polymer formulation under different buffer conditions, as explained in the key. (The figure was reproduced from [28••] with permission.)



DNA sequencing. It is possible that, even in the absence of the NIPA grafts and the high-temperature gelation they enable, the LPA backbone polymers alone might have provided comparable or better performance.

Buchholz *et al.* [29••] took another approach, designing polymers that exist as high-viscosity solutions (~3000 cP) at moderate sequencing temperatures (~45°C), but have a higher temperature viscosity switch driven by phase separation. These matrices are based on random copolymers of DMA and diethylacrylamide (DEA) with a DMA:DEA ratio of about 55:45. Upon heating to high temperatures (e.g. 70°C), the viscosity of these thermosensitive polymer solutions decreases by approximately two orders of magnitude and leads to greatly reduced microchannel loading time, as is seen in Figure 3. Here the viscosity drop occurs as the polymer solution phase-separates to form a colloidal dispersion, as shown schematically in Figure 4. This phase transition was shown to be fully reversible. Thus, the polymer matrix would be loaded into a microfluidic device at high temperature, and electrophoretic separation would be conducted at a lower temperature. The first generation of these thermoresponsive matrices provides DNA sequencing separations of 465–575 bases with 98.5% accuracy of base calling, depending on polymer formulation [29••]. The disadvantage of these matrices is that their relative hydrophobicity puts an upper limit on DNA sequencing read length [17]. For their implementation, they will require the development of microfluidic devices with dynamic spatial and temporal temperature control.

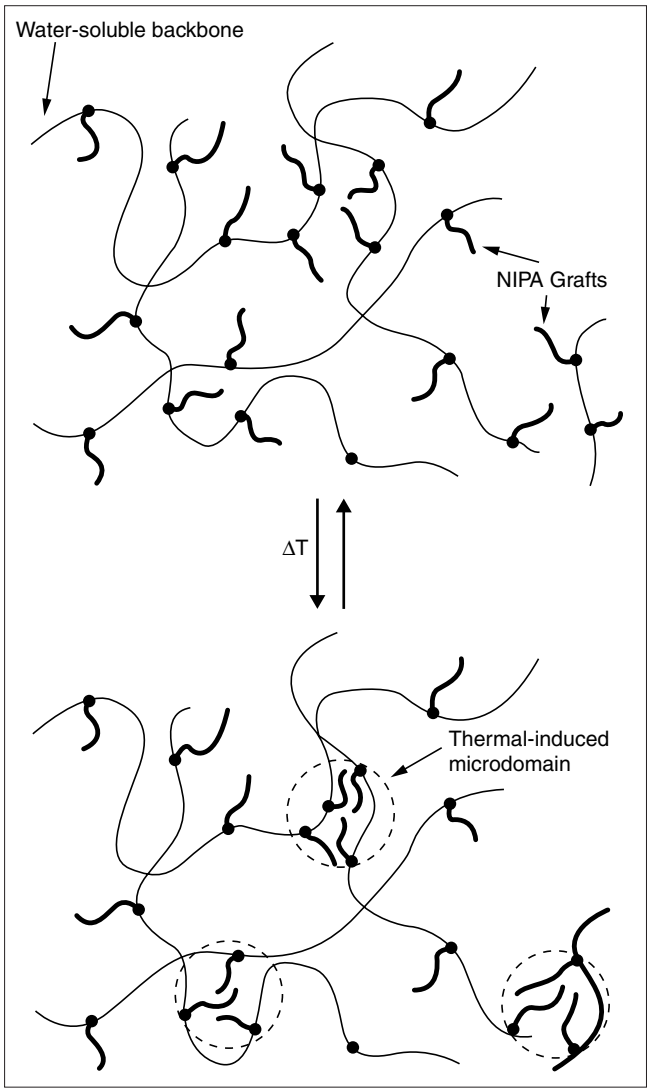
Another interesting approach to creating DNA separation matrices tailored for microfluidic devices was taken by Yeung's group [30••]. These researchers used nonionic surfactants of *N*-alkyl polyoxyethylene ethers to create worm-like micelles that associate to form a dynamic sieving network. By altering surfactant concentration, temperature, and denaturant concentration, the properties of the matrix (including micelle size and effective degree of micelle entanglement) can be adjusted for a particular DNA separation. By varying experimental parameters, dsDNA restriction fragment separations in the size range of 5000 base pairs and DNA sequencing separations up to 600 bases were achieved (although again no base-calling accuracy was given).

Wall-coating materials for microchannel systems

The second general class of functional materials of interest for microscale genomic and proteomic analysis is that designed to control the level of electro-osmotic flow (EOF) within the microchannel, and to eliminate any irreversible, nonspecific interaction of bioanalytes with the separation channel wall.

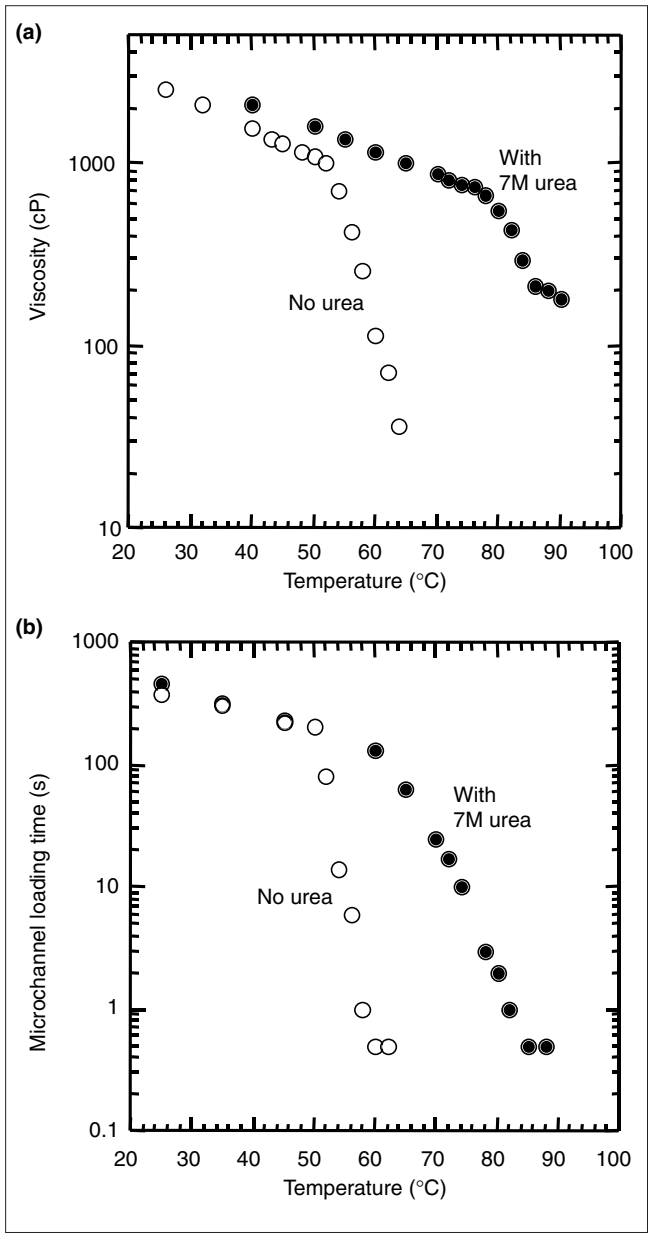
EOF is a phenomenon that occurs when an electrical double layer, generated by the attraction of soluble buffer ions to a charged surface on the separation wall, causes a local excess in the concentration of ions in the solution near the wall so that charge neutrality may be maintained proximal to the wall. The net result is that charged channel walls engender a bulk flow of fluid during electrophoresis. Depending upon the application, EOF in a microscale separation channel may be desired or not. If EOF is to be exploited towards positive ends, its magnitude should be uniform from one run to the next (and from one channel to the next) in a particular separation method. To meet this criteria, the effective charge density on the wall must remain stable over time. In practice, the velocity of EOF is extremely susceptible to drift and change if analytes become adsorbed to the walls, so maintaining EOF of a

Figure 2



A schematic illustrating the hypothesized mechanism of thermogelling, which results in a polymer solution whose viscosity is a binary function of temperature. (The figure was reproduced from [28••] with permission.)

Figure 3

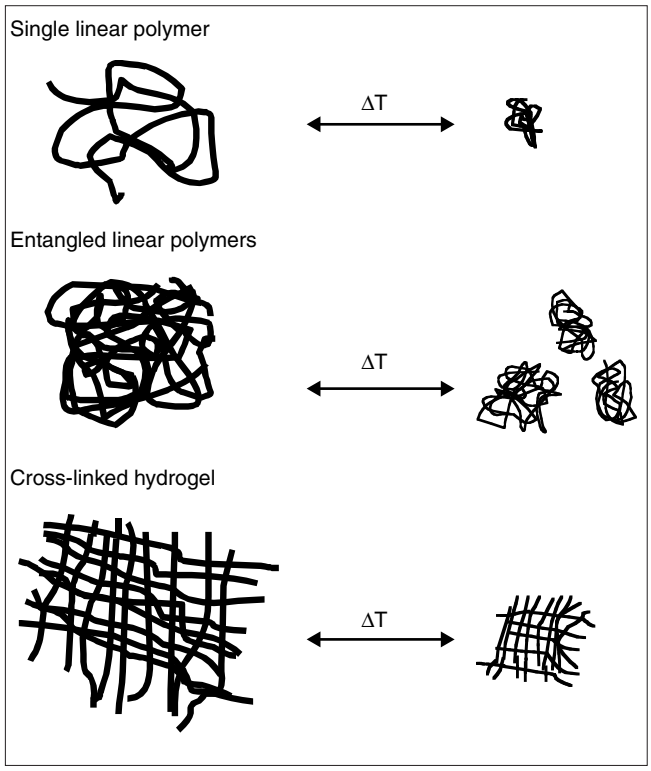


Plots showing the change in material properties of polymeric DNA sieving matrixes with a temperature-controlled viscosity switch. **(a)** Plot of viscosity versus temperature demonstrating the thermothinning properties of a thermosensitive polymer matrix that exists as a high viscosity solution at low temperature, and a low viscosity solution at elevated temperature. **(b)** Plot of loading time versus temperature demonstrating the ease of loading thermoreversible material in a low viscosity state. (The figure was reproduced in part from [29••] with permission. Copyright 2001 American Chemical Society.)

particular magnitude over time can be challenging. Finally, the chemical character of the separation channel wall coating needs to be such that any undesired, non-specific interactions with biological molecules are mitigated, including both hydrophobic and electrostatic interactions.

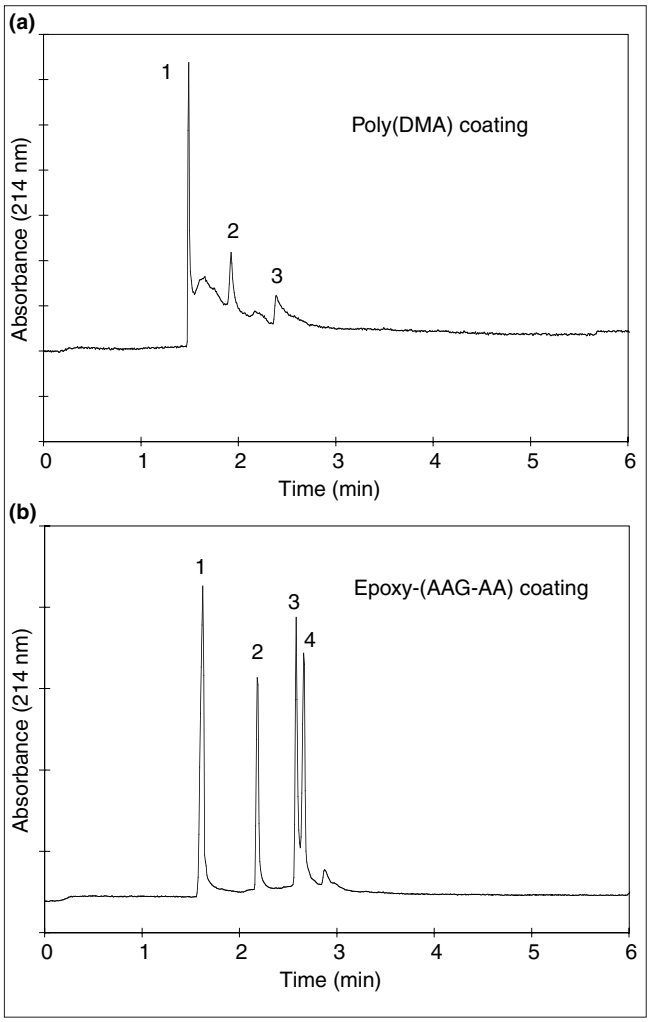
Wall coatings for microfluidic devices can be further divided into two categories by their method of application. The first type is 'dynamic' polymer or small-molecule coatings that adhere to the separation chamber wall via adsorbed physical interaction. This class of coatings has been reviewed recently [31]. The second type is covalently linked coatings, or coatings that are attached to the channel wall by covalent chemical bonds. Dynamic coatings are more desirable in that they are typically extremely fast and easy to create, usually requiring only a few minutes of channel rinsing with dilute solutions of the coating material. So far, however, these coating materials are of limited chemical diversity, lowering performance in some separations that demand an optimized coating chemistry. The covalently bound coatings are often much more laborious and time-consuming to form, requiring several distinct chemical reactions in series. However, these coatings can

Figure 4



A schematic illustrating the volume-phase transition of thermoreversible polymers, entangled polymer networks, and polymeric hydrogels. Entangled polymer networks exhibit a 'viscosity switch' in which phase transition leads to a lower viscosity state. As the temperature is increased, a single (dilute) linear polymer and entangled linear polymers undergo the collapse transition either as singular or as entangled chains. On the other hand, cross-linked hydrogels undergo the volume phase transition as single monolithic entities. (The figure was reproduced in part from [29••] with permission. Copyright 2001 American Chemical Society.)

Figure 5



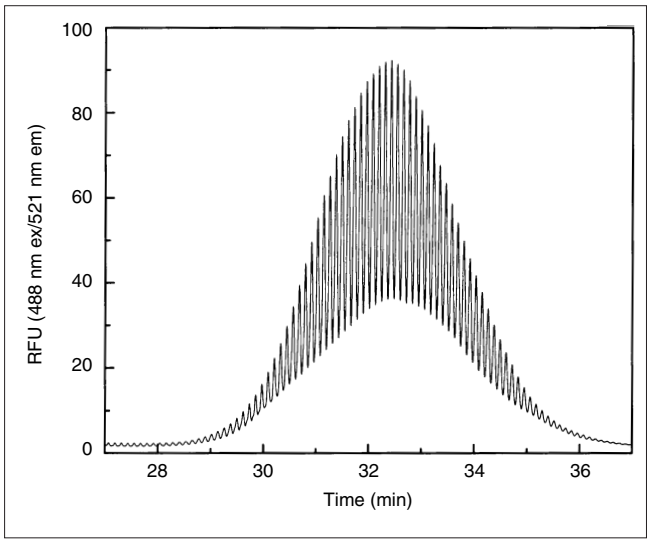
Capillary electropherograms demonstrating the impact of channel wall coating hydrophilicity on protein separation performance. **(a)** An electropherogram showing the separation of basic proteins (1–3) in a polyDMA-coated capillary. The separation is low-quality, owing to the hydrophobic nature of the capillary coating. **(b)** An electropherogram showing the separation of basic proteins (1–4) in an epoxy-AAG-AA-coated capillary. The separation quality is higher than for polyDMA, owing to the greater hydrophilicity of the capillary coating. (The figure was reproduced from [32•] with permission.)

often allow for greater chemical diversity, providing better performance in demanding applications, particularly protein analysis [32•].

Coatings for genomic analyses

High-performance DNA separations are usually optimally conducted in the absence of EOF; this requires neutral coating materials. Further, it is desirable to have a dynamic coating that obviates the need for complicated chemical coating procedures. Chiari and coworkers [32•] have recently introduced several new dynamic coatings that are easily employed in microscale separations. PolyDMA has long been known to form a stable adsorbed coating on fused silica and glass, but is of moderate hydrophobicity that can lead to nonspecific interactions with some analytes, particularly proteins. Moreover, the coating performance can degrade over the period of several runs, probably owing to surface fouling. In response to these challenges a copolymer of allylglycidyl ether and polyDMA — poly(dimethylacylamide-*co*-allylglycidyl ether) — was synthesized. The allylglycidyl ether portion of the copolymer presents an epoxy functionality, which is postulated to spontaneously form a covalent bond between the copolymer and the capillary wall [33]. In later work, a highly hydrophilic sugar-like monomer, allyl amide of gluconic acid, was incorporated into a copolymer with these other two monomers in an effort to create a more hydrophilic coating material, poly(acrylamide-*co*-allylamide of D-gluconic acid-*co*-allylglycidyl ether) (epoxy-AAG-AA). This material was suitable for both DNA and protein separations [27•]. When employed as a microchannel wall coating, the more hydrophilic epoxy-AAG-AA allows for much higher performance separations of basic proteins than does a polyDMA adsorbed coating, as seen in Figures 5a,b [32•].

Figure 6



An electropherogram showing the results of a free-solution conjugate electrophoresis analysis of a polyethylene glycol sample. This separation is enabled by the creation of a novel bioconjugate of ssDNA with polyethylene glycol. RFU (488 nm ex/521 nm em) is Relative Fluorescence Units with 488 nm excitation and 521 nm emission. (The figure was reproduced in part from [42••] with permission. Copyright 2001 American Chemical Society.)

Coatings for proteomic analyses

Typically, proteomic analyses in microfluidic devices are conducted with channel walls that are either neutral or positively charged. Neutral microchannel walls, for example coated with covalently bound LPA, are useful for protein separations in the absence of EOF. However, great care must be taken to avoid hydrophobic interaction between proteins and channel surfaces; proteins are notorious for adhering to glass, silica, and plastic [27•,32•,33]. As mentioned above, Chiari and colleagues [32•] have contributed much to this area.

Proteomic analyses are also conducted with positively charged microchannel walls, if the separation of proteins is conducted at low pH (below their isoelectric point) so that the proteins are predominately positive in charge. Under these conditions the added electrostatic repulsion between the wall and the analytes can mitigate undesirable interactions between these two entities. A recent paper in which multiple channels were used simultaneously required reproducible channel-to-channel EOF for accurate results [34]. An interesting coating, (*N*-methyl-*N*-ω-iodobutyl)-*N*′-methylpiperazine, was recently introduced and evaluated by Gelfi and coworkers [35,36••]. This molecule spontaneously bonds covalently to silica channel walls and allows EOF to be attenuated, eliminated or reversed simply by flushing the channel with different concentrations of the coating molecule. Perhaps most importantly, this coating material was shown to provide stable levels of EOF over several sequential protein analyses [36••].

Unique separation modalities made possible by bioconjugate materials

Some of the most interesting microfluidic separation techniques recently demonstrated were made possible by specifically designing and/or modifying the analyte (creating a novel bioconjugate) so that high-resolution electrophoretic separation could be achieved.

The analysis of trace small molecules in microfluidic systems can be challenging. However, Karger and coworkers [37•] showed that by producing a single-chain antibody via expression in *Escherichia coli* with a directly introduced fluorescent label, an affinity probe method could be used to detect the small molecule digoxin at 0.4 nM. Recombinant expression of the antibody creates an affinity probe that is electrophoretically homogeneous enough that upon binding to digoxin an easily detected mobility shift is introduced. Antibodies produced by more traditional techniques would have resulted in an electrophoretically heterogeneous sample, for which this mobility shift would not have been detectable.

One of the more promising separation modalities, end-labeled free solution electrophoresis (ELFSE), first postulated in a 1992 theoretical paper [38], has begun to come to fruition with the development of specialized materials (end-labels) required for the implementation of this separation mechanism. Attaching a monodisperse frictional entity to each of the DNA sequencing fragments in a Sanger mixture makes the mobility of DNA size-dependent in the absence of a sieving matrix. A few papers and reviews have shown the feasibility of this novel separation mechanism, which is especially amenable to microfluidic devices because viscous separation media are not required [39–41]. In an interesting twist on this idea, Vreeland *et al.* [42••] showed that uncharged polymers (polyethylene glycols) could be separated by electrophoresis of polymeric conjugates in free solution, if each individual polymer molecule is derivatized with an identical charged molecule (in this case DNA oligomers), as shown in Figure 6. Further, the resulting electropherograms could be used to determine the molecular mass distribution of the polymer sample. Upon the creation of larger frictional end-labels ELFSE will begin to live up to its theoretical promise to supplant gel-based DNA sequencing methods, and will allow a more elegant implementation of microfluidic devices for high-throughput genetic analyses.

Conclusions

Miniaturized chemical and biological analysis platforms promise to serve as critical enabling technologies for the burgeoning revolution in genomic and proteomic sciences. The design and fabrication of the hardware and detection systems for these devices is rapidly maturing, as evidenced by the number of groups publishing papers on novel fabrication methods. However, the design and characterization of the soft materials that allow optimal performance of miniaturized analysis systems currently lags behind the

hardware design. Relatively few groups have focused on the materials that will allow these systems to come to full fruition. Just as the astounding success of capillary electrophoresis in the human genome project was enabled by the design of materials (primarily sieving matrices) that allowed capillary systems to perform optimally, further miniaturized systems will require the design of materials specifically adapted for their unique features. The materials that engendered excellent performance in slab-gel electrophoresis were dismal failures in capillary-based systems. Similarly, materials that are designed for high-performance capillary-based systems will probably lead to suboptimal performance in microscale systems. Full realization of the high-throughput and cost-saving potential of microscale systems for proteomics and genomics will require the development of novel materials fashioned specifically for these systems.

Update

A paper by Albarghouthi *et al.* [43] has introduced a new polymeric sieving matrix for capillary electrophoresis of DNA, poly-*N*-hydroxyethylacrylamide, which is more hydrophilic than acrylamide and allows a DNA read length of up to 750 bases at 98.5% accuracy. Importantly, this polymeric separation matrix physically adsorbs to fused silica capillaries (like polyDMA) and minimizes EOF, thus obviating the need for a separate wall-coating material. Another study by Doherty and colleagues [44] investigated the adsorption behavior of *N*-alkyl-substituted polyacrylamides of varying hydrophobicities and characterized the level to which these polymers suppressed EOF. This information, as well as the thickness of the adsorbed polymeric coating on the silica surface, allowed the authors to show how polymer properties, including hydrophilicity and molar mass, contribute to adsorbed coating performance. For protein analysis, Lucy and coworkers [45] have introduced the use of phospholipid bilayers as dynamic adsorbed coatings. Finally, a new family of novel DNA–peptoid bioconjugate molecules was created by Vreeland, Slater and Barron [46], and analyzed by capillary electrophoresis with laser-induced fluorescence detection. Using a fluorescently labeled DNA oligomer as an electrophoretic 'engine', this method allows for the high-resolution separation of synthetic poly-*N*-substituted glycine (peptoid) oligomers produced by solid-phase organic chemical synthesis, and sensitive detection of contaminants in the sample. When the same oligomer preparations were analyzed via more traditional reversed-phase HPLC techniques, the contaminants were not detectable.

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