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Recent Advances in DNA Sequencing by End-Labeled Free-Solution Electrophoresis (ELFSE)

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Abstract End-Labeled Free-Solution Electrophoresis (ELFSE) is a new technique that is a promising bioconjugate method for DNA sequencing (or separation) and genotyping by both capillary and microfluidic device electrophoresis. Because ELFSE enables high-resolution electrophoretic separation in aqueous buffer alone (*i.e.*, without a polymer matrix), it eliminates the need to load viscous polymer networks into electrophoresis microchannels. To achieve microchannel DNA separations with high performance, ELFSE requires monodisperse perturbing entities (*i.e.*, drag-tags), which create a large amount of frictional drag when pulled behind DNA during free-solution electrophoresis, and which have other properties suitable for microchannel electrophoresis. In this article, the theoretical concepts of ELFSE and the required characteristics of the drag-tag molecules for the ultimate performance of ELFSE are reviewed. Additionally, the merits and limitations of current drag-tags are also discussed in the context of recent experimental data of ELFSE separation (or sequencing).

Keywords: DNA sequencing, capillary electrophoresis (CE), microfluidic device, ELFSE, drag-tag, microchannel electrophoresis

INTRODUCTION

Current state-of-the-art DNA sequencing technologies rely on electrophoresis in large arrays of fused silica capillaries filled with entangled polymer matrices (gel or polymer solution) to provide DNA separation. With these capillary array electrophoresis (CAE) instruments, National Human Genome Research Institute (NHGRI) and a private company, Celera Genomics, simultaneously released the working draft of the human genome in February 2001 [1,2], and both institutions declared that the sequencing of the entire human genome was essentially complete to high accuracy (*i.e.*, 99% or greater accuracy) in April 2003 [3]. CAE offers greater throughput than the previous generation of ultra-thin slab gel sequencers primarily because of the increased automation in the matrix- and sample-loading steps. As with semiconductor manufacturing, it seems that next generation of sequencing technology will be based on miniaturized electrophoresis systems ("microfluidic chips" or simply "microchips") because they have the clear potential to substantially increase the speed and throughput of automated DNA sequencing relatively to capillary array-based sequencing, while also reducing the cost per base [4-9]. However, microchannel electrophoresis, which includes both CAE and microchip electrophoresis, still has some difficult problems to overcome. Practically, one of the most difficult procedures in CAE is the introduction of the viscous polymeric sieving matrix into the capillary; this problem is even more obvious in capillary arrays and in ultra-miniaturized geometries such as chips [10].

DNA chains act like free-draining polymers during free-solution electrophoresis, so the polymeric sieving matrix is required to successfully separate DNA molecules of different sizes by electrophoresis [11]. Generally, as the concentration of high-molecular weight polymers in the sieving matrix increases (to a point), the 'read-length' (*i.e.*, the number of DNA bases that can be identified with certainty in a single electrophoresis run) increases. However, since the matrix viscosity also increases significantly with increasing polymer concentration, it is difficult to load high-performance sequencing matrices into microchannels. Thus, sequencing capability in a microchannel is a balance between read-length and the practical ability to load the polymer matrix. Although the Karger group attained a DNA sequencing read-length of 1,300 bases in a single CE run, before the analysis, they hand-loaded a 60,000 cP polymer solution into the capillary with a gas-tight syringe to provide several thousand psi to load the viscous polymer matrix [12]. In essence, then, this read-length cannot be accomplished with a high-throughput, automated CAE or microchip DNA sequencer. Pressure drop and flow rate depend strongly upon the channel diameter and the viscosity of the polymer solution: for laminar flow of a Newtonian fluid, the pressure gradient is $\Delta P/L = 128Q\eta / \pi d^4$ [13], where Q is volumetric flow rate, η is solution viscosity,

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and d is channel diameter. Consequently, loading a viscous polymer matrix into small microchannels is either very slow or requires very large pressure gradients.

Additionally, current DNA sequencing technology using polymeric sieving matrices has another theoretical performance limitation. The tendency of large DNA molecules to orient with the electric field during electrophoresis places an inherent limit on the read-length of ~1,000 bases for conventional polymer matrix-based sequencing [14]. Thus, to obtain a longer read-length in microchannel electrophoresis and/or to make microfluidic chips a practical replacement for CAE, it is necessary to develop a new sequencing method that allows long read-length without the use of high-viscosity sequencing matrices [15].

In 1992, Noolandi [16] first suggested that the constant charge-to-friction ratio of DNA that prevents its free-solution separation by electrophoresis could be overcome if DNA fragments were attached to a 'perturbing entity' such as a protein, virus, or microsphere. The only specified requirements of the perturbing entity were that it must be monodisperse (*i.e.*, that all perturbing entities are identical) and that it must have a different charge-to-friction ratio than DNA. This would allow the electrophoretic mobility of DNA to be a function of the DNA polymer chain length and provide for the separation of DNA in the absence of a sieving matrix [17,18]. This approach is known as *'End-Labeled Free-Solution Electrophoresis' (ELFSE)*, and this perturbing entity may also be called a *'drag-tag'* (see Fig. 1). The idea is that the drag-tag pulls behind the DNA during free-solution electrophoresis, reducing the electrophoretic mobility of the DNA by a fixed amount. If identical drag-tags are attached to all of the DNA molecules in an ensemble of differently sized ssDNA (single-stranded DNA) fragments, such as the sequencing ladder that results from the Sanger reaction [19], high-resolution DNA separation according to the number of bases can be achieved by electrophoresis in free solution.

In this article, the theoretical background of ELFSE and the required characteristics of drag-tag molecules optimally suited for ELFSE separation will be reviewed. Additionally, recent experimental results of ELFSE separation (or sequencing) will also be discussed.

ELFSE: THEORY AND CHARACTERISTICS

Theoretical Backgrounds

Electrophoresis is a technique that separates molecules according to differences in the velocity at which they migrate in an applied electric field. This migration velocity can be defined in terms of the electrophoretic mobility, μ (cm^2 $voltage^{-1}$ s^{-1}). At steady state, the electrophoretic mobility can be approximated as the ratio of the effective electrostatic charge, q (Coulomb), of the molecule of interest to the molecule's friction coefficient, f (kg/sec). It is also equal to the velocity of the analyte, V (cm/sec), divided by the electrical field, E (voltage/cm).

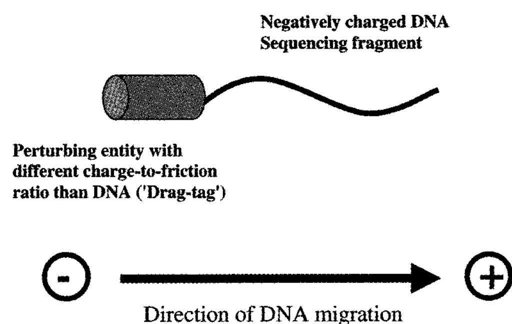

Fig. 1. Conceptual illustration of ELFSE.

$$\mu = \frac{q}{f} = \frac{V}{E} \tag{1}$$

Electrophoresis is the most common technique used to analyze DNA molecules. With a polymer backbone composed of negatively charged phosphate groups (pK_a~1.0), DNA is one of the most negatively charged biomolecules at physiological pH. The sugar groups and four bases represent a small percentage of the overall charge of the DNA polymer. On the other hand, the phosphate groups account for virtually all the charge of a DNA polymer. Their charge is independent of the sequence of DNA bases, and the overall charge is therefore essentially a linear function of the number of bases (or the polymer length). Sequence-dependent differences in the net charge of DNA molecules are generally only distinguishable for oligonucleotides shorter than 20 bases [20]. Thus, for large DNA:

$$q \sim N^1 \tag{2}$$

where N is the number of monomers in a DNA fragment. The negative charge of the DNA polymer backbone causes intramolecular electrostatic repulsion and forces DNA to adopt a highly expanded conformation in aqueous solution. Additionally, this negative charge causes a sheath of solvated positive ions to form around the polymer backbone. In the presence of an electric field, the DNA polyanions move towards to the anode, while positively charged counterions move towards to the cathode. The movement of this cation sheath in the opposite direction of the negatively charged DNA prevents hydrodynamic interaction between different segments of the DNA chain, and causes each monomer to contribute equally to the overall drag experienced by the DNA polymer. Thus, the friction coefficient is a linear function of the number of bases (or, the chain length) [21]. Hence, during electrophoresis in free solution, DNA behaves as a so-called 'free-draining coil':

$$f \sim N^1 \tag{3}$$

Since both the net charge and the molecular friction coefficient of the DNA are a linear function of the number of bases, the electrophoretic mobility of DNA is not a function of the number of bases in a particular DNA

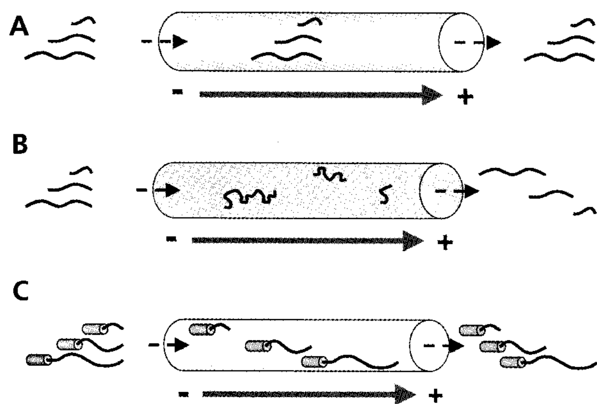

Fig. 2. Schematic representations of 3 different electrophoresis modes: (A) free-solution electrophoresis, (B) gel-based electrophoresis, (C) End-labeled free-solution electrophoresis (ELFSE).

fragment [22]. This is shown in the following equation:

$$\mu_{\mathrm{DNA}} = \frac{q}{f} \approx \frac{\rho(N^1)}{\xi(N^1)} = \frac{\rho}{\xi} = \mu_0 \quad (4)$$

where ρ is the charge of one DNA base, and ξ is the drag coefficient of one monomer of DNA during free-solution electrophoresis [17]. This results in all DNA fragments migrating at the same velocity during electrophoresis, and makes size separation of DNA by free-solution electrophoresis impossible (see Fig. 2A).

The inability to achieve size-dependent separation of DNA by free-solution electrophoresis required the implementation of different methods to make the analysis of DNA possible. In the mid-1960's, it was first discovered that DNA fragments can be separated by conducting electrophoresis in a porous medium, by a method known as a 'gel electrophoresis' [22]. The basic principle of gel electrophoresis is that DNA migration through a gel's interconnected network of polymer chains creates a nonlinear dependence of the friction coefficient on the DNA polymer length, thus allowing for size-dependent separation of DNA. Large DNA molecules experience a greater amount of friction than small fragments as a result of their greater configurational entropy, and therefore migrate through the gel more slowly (see Fig. 2B).

Recently, a new model for separating DNA, called ELFSE, has been generated. The physical mechanism behind ELFSE is based on the theory for the electrophoresis of polyampholytes developed by Long and coworkers and Slater *et al.* [17,23] and is outlined below. In this model, a DNA molecule with N monomers is conjugated to a drag-tag, and the drag-tag is said to have a net free-flow mobility equal to β times that of DNA in free solution (μ_0) and a total hydrodynamic drag equivalent to α bases of DNA. In the absence of segregation between the DNA and the drag-tag (such segregation could happen, for example, under extremely high fields), the new mobility of the DNA/drag-tag conjugate can be approximated as:

$$\mu(N) \approx \frac{q}{f} = \mu_0 \frac{(N-\beta)}{(N+\alpha)} \quad (5)$$

where μ_0 is the free-solution mobility of a normal free-draining DNA molecule. In essence, this equation assumes that the drag-tag behaves as a slightly different piece of DNA embedded in the random coil. Note the dependence on size (N) that has been introduced. If we plan to work with a neutral drag-tag ($\beta = 0$, for reasons that will be explained in the next section), the equation for mobility becomes simply:

$$\frac{\mu}{\mu_0} = \frac{1}{1+\alpha/N} \quad (6)$$

Since the elution time is inversely proportional to the net mobility, the difference in elution time for two consecutive DNA sequence peaks is given by:

$$\Delta t = (t_{\mathrm{N}} - t_{\mathrm{N+1}}) \sim \frac{\alpha}{N^2} \quad (7)$$

From this analysis we see the mobility increases with increasing N (the opposite of gel or matrix electrophoresis of DNA). This means that DNA fragments in a sequencing ladder will elute in the opposite order from standard gel electrophoresis: in ELFSE sequencing, the large fragments elute first, while the smallest fragments elute last (see Fig. 2C). As N becomes larger, the separation Δt between peaks becomes smaller. Thus, the value of α must be tailored to give an appropriate degree of separation, without slowing down DNA migration rates too much.

Required Characteristics of a Drag-tag Molecule

Below is a list of the requirements for a drag-tag molecule for ELFSE. The rationale for each requirement is discussed in detail in the text that follows.

- Uncharged (*i.e.*, $\beta = 0$) or slightly negatively charged
- Water-soluble (at pH 6~8)
- High frictional drag (*i.e.*, a large value of α)
- Homogeneous (monodisperse)
- Unique attachment to DNA sequencing primers by a high-yielding mechanism
- Minimal adsorption and non-specific interaction with the wall of the microchannel

Charge and Water Solubility

Mayer *et al.* proposed that the best drag-tag for ELFSE might be a protein or a virus particle [17]. Indeed, proteins produced by the biosynthetic machinery have the distinct advantage of being uniform in size and certainly can be quite large. However, most of the water-soluble proteins in nature contain both positively and negatively charged residues. A negatively charged protein is undesirable for use as a drag-tag because it would *increase* the

electrophoretic mobility of the hybrid molecule relative to an uncharged tag, lessening the effective separation. On the other hand, a positively charged protein may at first seem to be desirable because it would *decrease* the electrophoretic mobility of the hybrid molecule. However, the use of a positively charged drag-tag would actually be detrimental to the efficient separation of DNA fragments. Electrostatic interactions between DNA and a positively charged moiety would disturb the native conformation of the DNA. In this state, DNA would no longer be a free-draining coil, and, consequently, the friction coefficient (f) of DNA would not be proportional to the chain length. Furthermore, positively charged proteins would be likely to bind to the negatively charged microchannel walls. For these reasons, the ideal drag-tag will be uncharged (*i.e.*, $\beta = 0$). A slight negative charge could be tolerated, however.

Even if the overall charge of a water-soluble protein were near zero at pH 7~8 (*i.e.*, if the protein was net-neutral), generally several positive amino acids and negative amino acids may be evenly distributed throughout this protein. In this case, local electrostatic interactions could also occur during free-solution electrophoresis. Thus, a natural water-soluble protein can be said to be suboptimal for use as a drag-tag molecule, because it will likely present a chemically 'patchy' (either charged or hydrophobic) surface. Instead, a synthetic polypeptide composed of hydrophilic and uncharged amino acids used as a drag-tag molecule will likely allow for optimal ELFSE performance.

Frictional Drag Coefficient (α value)

It can be shown that a high frictional drag coefficient (*i.e.*, high α value) is absolutely necessary for long-read DNA sequencing. That is, to separate large DNA fragments successfully, a drag-tag must exhibit a high α value. Suppose that we are concerned with electrophoretically separating two hybrid molecules containing DNA fragments of 1,000 and 1,001 bases. If we also consider two different drag-tags with $\beta = 0$ and α values of 100 and 200, respectively, then the electrophoretic mobility of a hybrid molecule bearing a neutral label is given by:

$$\mu(N) = \mu_0 \frac{N}{(N+\alpha)} \quad (8)$$

And $\Delta\mu$, the difference in mobility of the two hybrid molecules is given by:

In the case of $\alpha = 100$: (9)

$$\Delta\mu = \mu_0 \left[\frac{1001}{(1001+100)} - \frac{1000}{(1000+100)} \right] = 8.257 \times 10^{-5} \mu_0$$

In the case of $\alpha = 200$: (10)

$$\Delta\mu = \mu_0 \left[\frac{1001}{(1001+200)} - \frac{1000}{(1000+200)} \right] = 1.388 \times 10^{-4} \mu_0$$

As shown in Eqs. (9) and (10), a drag-tag with a higher α value has a higher resolving power for DNA separation as evidenced by the greater difference in μ. Therefore it is important that the α value be as large as possible (up to ~1,000) so that the increasingly smaller changes in μ caused by increases in the number of bases in the sequencing fragments are electrophoretically discernable.

Drag-tag Monodispersity

In practice, drag-tag monodispersity is one of the most difficult characteristics to successfully create in an ELFSE drag-tag. In order to form a sharp peak, each of the individual DNA fragments must experience the same amount of drag. An ideal drag-tag molecule should therefore be composed of exactly the same number and sequence of monomers. If this is not the case, then the electrophoretic mobility of each individual sequencing fragment can be influenced by a different α value. As a result, a DNA fragment of a given length would exhibit a range of mobilities according to the heterogeneity of the drag-tags, thus obscuring the DNA sequencing results.

Attachment of DNA to the Drag-tag

To implement ELFSE successfully, the drag-tag must have a single, unique chemical or biophysical functionality that allows it to be attached to DNA fragments. The attachment of the DNA to the drag-tag may be based on either biophysical binding or covalent linkage. Ren *et al.* demonstrated that ssDNA fragments from a Sanger reaction could be separated in free solution using the natural, folded protein, streptavidin, as a drag-tag [24]. To accomplish this, this group performed the cycle sequencing reaction with DNA primers that had a 5' biotin modification. After the cycle sequencing reaction, purified streptavidin was bound to the biotin functionality, creating a stable linkage between the DNA fragment and the drag-tag (the dissociation constant of streptavidin-biotin is $\sim 10^{-15}$ M). When these labeled ssDNA fragments were subjected to CE in a 25-μm i.d. capillary, in a buffer containing 3 M urea, this system allowed for the reading of ~110 bases of DNA sequence.

Although these results demonstrated the potential for DNA sequencing using ELFSE, in general, the biophysical binding between a polypeptide and a DNA fragment is not stable under the strongly denaturing conditions of DNA sequencing electrophoresis (typically 7 M urea and 55°C). These denaturing conditions are used to prevent biophysical intra-strand interactions in individual DNA strands; as a result, any physical conjugation mechanism will require a high binding affinity to ensure the stability of the bond under such conditions. Another drawback of biophysical binding is that there is no universal conjugation method that can be applied to a typical protein. Streptavidin, used in these experiments, is a unique protein with its strong binding affinity for biotin.

To ensure that the drag-tag remains attached to the DNA fragments throughout DNA sequencing electrophoresis and to allow any protein to be used as a drag-tag, a covalent attachment is preferred for binding the drag-tag to the DNA fragments. One chemical strategy takes ad-

vantage of maleimide-thiol chemistry [25,26]. In this approach, the maleimide functionality is placed on the drag-tag's amino terminus. If DNA primers that are terminated at their 3′ ends with a thiol (-SH) group are then used in the cycle sequencing reaction, the DNA fragments can be conjugated to the drag-tag because maleimides react quantitatively with thiols in moderate to basic aqueous solutions.

EXPERIMENTAL RESULTS OF ELFSE

ELFSE Separation with Streptavidin as a Drag-tag

In the time since the ELFSE concept was first proposed and examined on a theoretical basis in the early 1990s [16,17], many researchers have subsequently shown that this bold proposal holds promise for the separation of oligonucleotides [27,23] and oligosaccharides [28,29]. However, ELFSE for the separation of large DNA fragments was not validated until 1998 [30]. Prior to this report, difficulties related to selecting the proper label, attaching it to the DNA fragments, and avoiding interactions with the capillary walls were thought to be intractable. Heller and co-workers used streptavidin, a natural protein, as a drag-tag for a double-stranded DNA mixture, due to its near-zero electrical charge and the fact that it can easily be attached to DNA via a biotin complex. Streptavidin is a tetramer of 159-residue polypeptide chains, and is well known for its strong binding to biotin. Using this label, the separation of 100-base dsDNA ladder (up to ~1,000 bases) was achieved in free-solution CE [30]. However, the resulting peaks were broad, and the resolution of DNA separation was about 10 bases in the best case, clearly inadequate for DNA sequencing.

In 1999, Ren and co-workers performed the first demonstration of ELFSE sequencing using gel-purified streptavidin as a drag-tag [24]. Since streptavidin typically shows considerable heterogeneity at the amino- and carboxyl-termini of each subunit due to differential proteolysis during biosynthesis and secretion [31], the streptavidin protein was purified through a polyacrylamide gel column to improve protein homogeneity. With an improved wall-coating agent (*i.e.*, POP6) and greater monodispersity in the streptavidin label, they were able to sequence up to ~110 bases in 18 min. Although these remarkable results provided the first demonstration of ELFSE sequencing, they also showed some of the limitations of natural proteins for sequencing drag-tags. Obviously, the read-length of the DNA sequencing reaction obtained with streptavidin-based ELFSE is far from being competitive with that of conventional gel or polymer matrix-based electrophoresis; the typical read-length of matrix-based electrophoresis with an ABI PRISM 3730™ is ~700 bases at 99% accuracy in 1 h [26]. According to their analyses, analyte-wall interactions are responsible for most of the decrease in resolution at high electric field, and these interactions could be minimized by using labels that are specially designed to be less adsorptive and, at the same time, generate friction. Additionally, streptavidin has a small α value due to its globular conformation (the measured α value of streptavidin is only 30 [24]). As larger α values increase the resolution of ELFSE separation, the obvious way to separate larger DNA fragments is to use a larger friction-generating label. Based on both the physical equations they derived as well as experimental data, Ren *et al.* predicted that a read-length of about 625 bases could be obtained with α = 300, E = 1,000 V/cm, and L (channel length) = 40 cm; more recently Slater *et al.* have predicted that 1,300 bases could be sequenced in 700 sec (<12 min), under optimized conditions with a drag-tag having α = 400, E = 2,000 V/cm, and L = 20 cm [26].

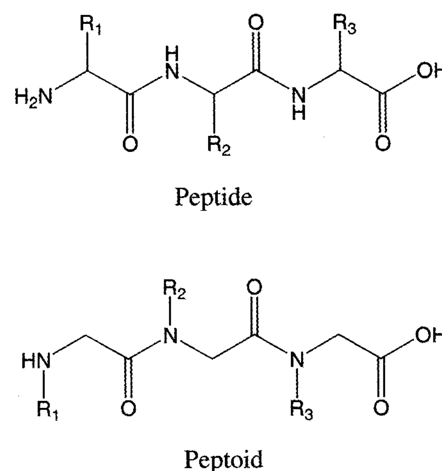

Fig. 3. A comparison of the structures of peptide- and peptoid-trimers with arbitrary side-chains (R_1, R_2, and R_3).

ELFSE Separation with Non-natural Drag-tags: Polypeptoid Drag-tags

Since ELFSE results with streptavidin illustrated that natural proteins have significant limitations as drag-tags, synthetic polypeptides have been studied as new drag-tag candidates. Polypeptoids, or poly-*N*-substituted glycines, are a novel class of synthetic molecules that are quite similar to polypeptides [32,33], and these molecules can also serve in generating suitable drag-tags. Like polypeptides, polypeptoids are based on a peptide backbone. However, in polypeptoids the side-chains are appended to the amide nitrogen rather than to the α-carbon, as shown in Fig. 3. This simple structural alteration leads to many changes in the biophysical behavior of this molecule, including higher water-solubility and side-chain diversity.

Recently, a set of uncharged, hydrophilic polypeptoids, produced using an automated peptide synthesizer and purified to monodispersity by HPLC, were conjugated to 20- or 21-base oligonucleotides through a maleimide-thiol conjugation reaction, and the polypeptoid-oligonucleotide conjugates were then analyzed via capillary electrophoresis [25]. In addition, high friction-generating, comb-like graft copolymers, created by attaching these

polypeptoids to a polypeptide backbone, were used as drag-tags with high resolution by ELFSE [34]. The results of these experiments suggest that polypeptoids generated via solid-phase synthesis are useful for the separation of small oligonucleotides such as 20 or 30 bases, but are not applicable to DNA sequencing with long reads because they are too small (the measured α values of the comb-like graft copolymers range from 8 to 17 [34]). In order to separate large DNA fragments (*e.g.*, >150 bases), a drag-tag with large frictional drag is essential. However, there is a limit to the practicality of organic synthetic methods for the production of long, monodisperse polypeptides or polypeptoids [26].

ELFSE Separation with Synthetic Polymer Drag-tags

Chemically synthesized polymers such as polyethylene glycol (PEG) are not suitable as drag-tags for high-resolution DNA separations, because they are inevitably polydisperse in terms of molecular weight distribution. Even PEG with a polydispersity index (M_w/M_n) of only 1.01 (highly monodisperse by synthetic polymer standards) yielded a large cluster of ~110 close-spaced peaks or a broad smear, for each size of DNA in free-solution capillary electrophoresis [35]. This polydispersity renders any known synthetic polymer made by solution-phase methods ineffectual for high-resolution DNA sequencing separations [36].

ELFSE Separation with Repetitive Polypeptide Drag-tags

Since most synthetic polymers and natural proteins (such as streptavidin) are unsuitable for DNA sequencing by ELFSE based on the variety of reasons discussed above, non-natural repetitive polypeptides (or 'protein polymers') have recently been studied as novel drag-tags. Protein polymers are highly repetitive polypeptides produced by genetic engineering technology [37]. These protein polymers offer significant advantages for ELFSE application over other drag-tag candidates, in that their sequences and lengths are controllable, not only to generate monodispersity and high frictional drag, but also to meet other drag-tag requirements for microchannel electrophoresis. Although this method requires biological protein expression, a more complicated and lengthy procedure for obtaining the final polypeptides than chemical synthesis, it can provide a monodisperse and long protein polymer with the desired molecular properties. In order to produce protein polymers with desirable properties, well-defined cloning methods for concatemer genes (or multimer genes) should be followed. Recursive directional ligation (RDL) method [38] and controlled cloning method [39] are examples of cloning methods that were developed to allow the construction of large concatemers of repetitive DNA sequences. Won and colleagues used the controlled cloning method to generate these large, repetitive genes, which were then transformed into *E. coli* and expressed. After purification, the protein polymers were conjugated to DNA via a coupling linker like sulfo-SMCC and analyzed by CE. Experimental results indicated that non-natural protein polymers are much more promising candidates for ELFSE drag-tags, although the repetitive polypeptide sequences produced thus far have suffered from unforeseen drawbacks, including the deamidation of glutamine and the consequent unacceptable polydispersity [26]. Therefore, neutral protein polymers with no unstable glutamine residues are currently under development, and it is anticipated that the ELFSE technique should provide an attractive alternative to matrix-based DNA sequencing as more homogeneous and higher-friction protein polymer drag-tags are developed.

CONCLUDING REMARKS

End-Labeled Free-Solution Electrophoresis (ELFSE) is a promising DNA sequencing technology for both capillary array instruments and microfabricated devices because it eliminates the need for loading viscous sequencing gel into the microchannels. Additionally, ELFSE provides better results at high voltages as it does not require a sieving matrix, and this subsequently leads to faster separation. Therefore, ELFSE could become the separation method of choice for fast sizing of DNA fragments in mapping and diagnosis applications. Although ELFSE-based DNA separation has still read-length limitations, the limitations faced in this mode of separation are fundamentally different than those faced in gel-based electrophoresis, which were predicted theoretically by Slater *et al.* [14]. Developing larger monodisperse drag-tags, contriving highly efficient sample injection methods, eliminating the analyte-wall interaction, and applying higher electric fields will extend ELFSE's read-length [24]. Many research groups are currently pursuing the development of this new DNA sequencing method enabled by ELFSE, and it is expected these efforts will succeed in providing longer read-length and faster separations than current electrophoretic systems based on polymer-sieving matrices.

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