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Research Article

Free-solution electrophoresis of DNA modified with drag-tags at both ends

In end-labeled free-solution electrophoresis (ELFSE), DNA molecules are labeled with a frictional modifier or "drag-tag", allowing their size-based electrophoretic separation in free solution. Among the interesting observations from early work with dsDNA using streptavidin as a drag-tag was that the drag induced by including a streptavidin label at both ends was significantly more than double that from a single streptavidin (Heller, C. *et al.*, *J. Chromatogr. A* 1998, *806*, 113–121). This finding was assumed to be in error, and subsequent work focused on experiments in which only a single drag-tag is appended to one end of the DNA molecule. Recent theoretical work (McCormick, L. C., Slater, G. W., *Electrophoresis* 2005, *26*, 1659–1667) has examined the contribution of end-effects to the free-solution electrophoretic mobility of charged-uncharged polymer conjugates, reopening the question of enhanced drag from placing a drag-tag at both ends. In this study, this effect is investigated experimentally, using custom-synthesized ssDNA oligonucleotides allowing the attachment of drag-tags to one or both ends, as well as dsDNA PCR products generated with primers appropriate for the attachment of drag-tags at one or both ends. A range of sizes of drag-tags are used, including synthetic polypeptoid drag-tags as well as genetically engineered protein polymer drag-tags. The enhanced drag arising from labeling both ends has been confirmed, with 6–9% additional drag for the ssDNA and 10–23% additional drag for the dsDNA arising from labeling both ends than would be expected from simply doubling the size of the drag-tag at one end. The experimental results for ssDNA labeled at both ends are compared to the predictions of the recent theory of end-effects, with reasonably good quantitative agreement. These experimental findings demonstrate the feasibility of enhancing ELFSE separations by labeling both ends of the DNA molecule, leading to greater resolving power and a wider range of applications for this technique.

Keywords: DNA sequencing / Drag-tags / End-labeled free-solution electrophoresis / Genotyping / Molecular end-effect DOI 10.1002/elps.200500554

1 Introduction

1.1 General

Size-based separations of DNA for applications such as DNA sequencing and genotyping are frequently accomplished by electrophoresis in a polymeric sieving matrix, examples of which include crosslinked gels and highly entangled solutions of linear polymers [1]. Although this technique is a workhorse of modern molecular biology, the sieving matrix imposes limitations on the speed of separation, and electric field-induced band-broadening and molecular orientation effects lead to a reduced ability to separate larger DNA fragments [1–4]. Additionally, crosslinked gels and viscous polymer solutions are problematic to load into miniaturized microfluidic devices currently being developed for DNA sequencing, PCR product sizing, and other electrophoretic separations [5–9].

A variety of alternative DNA separation modes have been proposed for use in capillaries and microfluidic devices [10], including entropic trapping [11, 12], separation in ultradilute polymer solutions [13] or in microfabricated

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Abbreviations: ELFSE, end-labeled free-solution electrophoresis; **NMEG**, *N*-methoxyethylglycine; **Sulfo-SMCC**, sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate; **TCEP**, tris(2-carboxyethylphosphine)

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arrays of posts or other obstacles [14, 15]. One exciting approach that has received considerable attention is end-labeled free-solution electrophoresis (ELFSE) [16–20]. In this approach, DNA is modified end-on with an uncharged, monodisperse, polymeric end-label, or "drag-tag" to create a charged-uncharged polymer conjugate. During electrophoresis in free solution, the drag-tag imparts the bioconjugate with a fixed amount of additional hydrodynamic friction. The additional friction modifies the electrophoretic mobility of the DNA-drag-tag conjugates in a size-dependent fashion: Conjugates comprising small DNA fragments migrate more slowly than conjugates with large DNA fragments, and thus a size-based separation can be accomplished in the absence of a sieving matrix.

The theoretical principles and experimental demonstrations of ELFSE have been recently reviewed [20]. In the first experimental demonstration of ELFSE, streptavidin was used to label dsDNA restriction fragments that had been biotinylated at one or both ends [18]. The efficiency of this separation was limited primarily by the inherent polydispersity of the streptavidin label, as well as by interactions between the streptavidin and the capillary walls. One of the interesting results of this study, however, was that the amount of hydrodynamic drag associated with adding a streptavidin label to both ends of the DNA was observed to be significantly more than twice the friction for adding streptavidin to one end only. Whereas a single streptavidin provided friction equivalent to an additional 23 bp of DNA, two streptavidins provided the friction of an additional 54 bp, 17% greater than would be expected from simply doubling the amount of friction from a single streptavidin. The implications of this finding were not fully appreciated at the time, and, being attributed to experimental error, this effect was not explored further.

In later work, a gel-purified streptavidin was used to label ssDNA sequencing fragments generated using a 5'-biotinylated primer [19]. Using the more homogeneous streptavidin as a drag-tag at the 5' end of the sequencing fragments, and employing a more effective wall-coating agent, approximately 110 bases of the four-color sequencing reaction were separated by ELFSE. Although these initial results were promising, the main limitation preventing the further use of ELFSE has been the lack of suitable large, water-soluble, monodisperse drag-tags with appropriate chemical functionality for unique attachment to DNA. More recently, progress has been made with the development of novel drag-tags consisting of long, repetitive, genetically engineered polypeptides (or "protein polymers") [21, 22], or linear or branched polyamides synthesized by solid-phase techniques [23–25]. A variety of these new drag-tags have been used in this study to revisit the potential for performing ELFSE separations of DNA molecules with drag-tags at each end.

1.2 Theory of end-effects in ELFSE

The standard theory of ELFSE was developed through investigations into the electrophoretic mobility of polymers with nonuniform charge distributions. For the case of the migration of a DNA-drag-tag conjugate, with a charged DNA segment consisting of M_C charged monomers and an uncharged drag-tag consisting of M_U uncharged monomers, the mobility μ is given by a weighted average of the electrophoretic mobilities of the charged and uncharged monomers:

$$\mu = \mu_0 \frac{M_C}{M_C + \alpha_1 M_U} \tag{1}$$

where μ_0 is the mobility of the charged monomers (*i.e.*, the free-solution mobility of DNA). (The uncharged monomers have zero electrophoretic mobility, and thus do not appear in the numerator of Eq. (1).) The parameter α_1 reweights the number of uncharged monomers M_U to reflect differences in persistence length and other hydrodynamic properties. The product $\alpha_1 M_U$, referred to as α, describes the total friction provided by the drag-tag, in terms of the number of additional uncharged monomers of DNA that would add equivalent friction. Thus, in the experiments described previously [18], a single streptavidin drag-tag provided $\alpha = 23$, *i.e.*, an amount of friction equivalent to 23 uncharged bp of DNA, whereas two streptavidins gave $\alpha = 54$. Notably, Eq. (1) cannot adequately explain the more than doubling of α arising from using two drag-tags.

The weighting of the individual monomer units in constructing the average in Eq. (1) was recently re-examined theoretically [26]. Whereas previous theory assumed that each monomer unit (after rescaling the uncharged monomers by α_1) contributes equally to the electrophoretic mobility of the composite molecule, more recent theory has taken into account end-effects originally described by Long *et al.* [27]. According to this theory, monomer units near either end of the polymer chain have greater influence than monomer units near the middle in determining the electrophoretic mobility of the composite molecule. This can be expressed by including a weighting factor ψ in the calculation of the mobility. For the case of ELFSE, with M_C charged monomers conjugated end-on to M_U uncharged monomers, and scaling M_U by the factor α_1 such that the total number of monomers is effectively $N = M_C + \alpha_1 M_U$, the weighted average mobility is expressed as

$$\mu = \frac{1}{N}\int_0^{M_C} \mu(n)\Psi\left(\frac{n}{N}\right)dn \quad (2)$$

where the index of integration, n, represents the position of a charged monomer unit in the chain. The ratio n/N, which appears as the argument of the weighting function ψ, ranges from 0 to 1, and represents the relative position of a given monomer unit in the chain. The limits of integration are written from 0 to M_C (rather than 0 to N) since the uncharged monomers ($n = M_C + 1 \ldots N$) have zero electrophoretic mobility, and only the charged monomers contribute to the total. Making the further substitution that for charged DNA monomers, the mobility $\mu(N) = \mu_0$, and using the definition $N = M_C + \alpha_1 M_U$, the mobility of the composite molecule can be written as

$$\mu = \frac{\mu_0}{M_C + \alpha_1 M_U}\int_0^{M_C} \Psi\left(\frac{n}{M_C + \alpha_1 M_U}\right)dn \quad (3)$$

The normalized weighting function $\psi(n/N)$ of a Gaussian polymer chain was found in [26] to be well represented by the following function:

$$\Psi\left(\frac{n}{N}\right) \approx -0.65 + 0.62\left(\frac{n}{N}\right)^{-\frac{1}{4}} + 0.62\left(1 - \frac{n}{N}\right)^{-\frac{1}{4}} \quad (4)$$

Equation (4) is a well behaved, easily calculated (and easily integrated) function for $0<(n/N)<1$, and is depicted in Fig. 1 of [26]. Using this functional form in Eq. (3) allows the straightforward calculation of the electrophoretic mobility for any composite molecule consisting of a DNA chain linked end-on to an uncharged drag-tag chain, provided that the scaling factor α_1 is known for a given set of experimental conditions. For the slightly more complicated case of a charged DNA chain with uncharged drag-tags at *both ends* of the DNA chain, Eqs. (2) and (3) need only be modified by changing the limits of integration, and the total number of effective monomer units N. For the case of a DNA chain consisting of M_C charged monomers, with identical drag-tags consisting of M_U uncharged monomers at each end, the total number of effective monomers is now $N = M_C + 2\alpha_1 M_U$. With this change, and inserting the appropriate limits of integration, the mobility becomes

$$\mu = \frac{\mu_0}{M_C + 2\alpha_1 M_U}\int_{\alpha_1 M_U}^{\alpha_1 M_U + M_C} \Psi\left(\frac{n}{M_C + 2\alpha_1 M_U}\right)dn \quad (5)$$

Besides providing a more complete analysis of the electrophoretic mobility of ELFSE conjugates, and improving the quantitative analysis of previous data from the molar mass profiling of PEG [28], the theory of end-effects makes useful predictions for enhancing the performance of DNA sequencing and other separations using ELFSE. The $\psi(n/N)$ function in Eq. (4) has its maxima near the ends of the molecule, indicating that the chain ends are

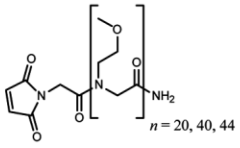
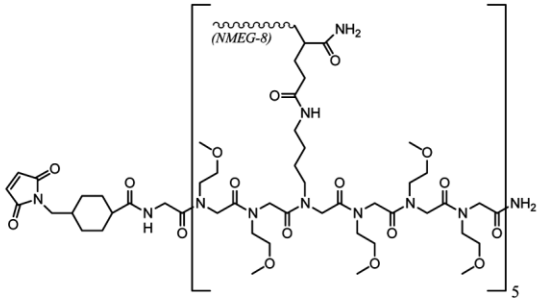
Drag-tag	Structure	References
NMEG-20 NMEG-40 NMEG-44		[23-25]
Branched NMEG-70		[31]
P1-169	H_2N - (Gly Ala Gly Gln Gly Ser Ala)$_{24}$ Gly - COOH	[21, 22]
P2-127	H_2N-(GAGTGSA)$_4$-GAGTGRA-(GAGTGSA)$_7$-GAGTGRA-(GAGTGSA)$_5$-G-COOH	[21]

Figure 1. Structures and code names for the six different drag-tag molecules used in this study. P1–169 and P2–127 drag-tags had maleimide functionalites added to their N-termini by activation with sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate (Sulfo-SMCC), as described in [22].

weighted more heavily in determining the electrophoretic mobility of the composite molecule. The heavier weighting of the chain ends implies that adding an uncharged drag-tag to each end of a DNA molecule provides more than twice the drag of using a single drag-tag of the same size at one end of the DNA molecule. This is consistent with the initial experimental observations using streptavidin as a drag-tag [18]. Moreover, since the production of very large, totally monodisperse drag-tag molecules has thus far been problematic [22, 29], the effect might be exploited to provide sufficient drag for high-efficiency separations by using two smaller (and more monodisperse) drag-tags, rather than one larger drag-tag. In this study, we provide experimental confirmation of this effect using both short ssDNA oligos and larger dsDNA PCR products, with drag-tags of varying sizes at one or both ends of the DNA molecules.

2 Materials and methods

2.1 Chemicals

Tris(2-carboxyethylphosphine) (TCEP) and maleimide were purchased from Acros Organics (Morris Plains, NJ, USA). Sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Pierce (Rockford, IL, USA). Buffer salts Tris (free base), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), and EDTA were purchased from Amresco (Solon, OH, USA). POP-6 polymer solution was purchased from Applied Biosystems (Foster City, CA, USA). All water was purified using an E-Pure system from Barnstead (Boston, MA, USA) to a minimum resistivity of 17.8 MΩ·cm.

2.2 Drag-tag molecules

Six different drag-tag molecules were used in this study. Three were linear *N*-methoxyethylglycine (NMEG) oligomers of length 20, 40, or 44 monomers, produced by a solid-phase submonomer synthetic protocol [30], capped with an *N*-terminal maleimide, and purified to monodispersity by RP-HPLC as described previously [23–25]. Another drag-tag used was a monodisperse branched molecule consisting of a 30mer poly(NMEG) backbone with five octamer oligo(NMEG) branches, also described previously [31]. The final two drag-tags were repetitive protein polymers of length 127 and 169 amino acids, produced using the controlled cloning technique [21], and activated at the *N*-termini using the heterobifunctional cross-linker Sulfo-SMCC by reacting the protein polymers with a ten-fold molar excess of Sulfo-SMCC for 1 h at room temperature and pH 7.2, and then removing excess cross-linker by gel filtration as described previously [22, 29]. The structures and short names of the drag-tags are shown in Fig. 1. The NMEG-20 and NMEG-40 drag-tags were used for the studies of ssDNA, whereas the larger tags were used for the studies of dsDNA. All of the drag-tags used are hydrophilic, water-soluble molecules. Following the maleimide activation of the *N*-termini, the NMEG drag-tags are charge-neutral, whereas the P1–169 has a net charge of –1 (from deprotonation of the C-terminus), and the P2–127 (with two cationic arginine residues) has a net charge of +1.

2.3 Production of ssDNA conjugates

Two poly(dT) oligonucleotides of length 20 and 40 bases were purchased from Integrated DNA Technologies (Coralville, IA, USA). The oligos were modified at the 5'-end with a thiol linker that has a 6-carbon spacer, and at the 3'-end with a thiol linker having a 3-carbon spacer. The oligos were also modified internally with a fluorescein-dT base near the middle of the chain. These dithiolated, fluorescently labeled oligos (referred to as T20-dithiol and T40-dithiol) are shown schematically in Table 1.

Table 1. Oligonucleotides used for producing ssDNA conjugates with drag-tags at one or both ends

Oligonucleotide	Sequence
T20-dithiol	X_1 TTTTTTTTTX_2 TTTTTTTTT X_3
T40-dithiol	X_1 TTTTTTTTT TTTTTTTTTX_2 TTTTTTTTT TTTTTTTTT X_3

X_1, 5'-thiol linker with 6-carbon spacer; X_2, internal fluorescein-dT base; X_3, 3'-thiol linker with 3-carbon spacer

The thiol linkers on the DNA oligos were reduced using TCEP. To accomplish this reduction, 400 pmol of the dithiolated ssDNA (either T20-dithiol or T40-dithiol) was mixed with a 40:1 molar excess of TCEP, in a total volume of 10 μL of sodium phosphate buffer (100 mM, pH 7.2). This mixture was incubated at 40°C for 2 h. The reduced DNA was then split into aliquots of 10 pmol each prior to the addition of the drag-tag. To one aliquot, a large excess of maleimide (5 nmol) was added, capping the reduced thiols, and creating ssDNA molecules with no drag-tag (except the maleimide). To another aliquot, a large excess of drag-tag (1 nmol of either NMEG-20 or NMEG-40) was added, such that the majority of ssDNA molecules would have polymeric drag-tags at both ends. The other aliquots were treated with different amounts of drag-tag, from 50 to 200 pmol, with the intent of creating mixtures containing appreciable amounts of DNA with zero, one, or

two drag-tags. After reacting for approximately 90 min, an excess of maleimide (5 nmol) was added to these reactions to cap any remaining free thiols. The reactions were incubated in the dark at room temperature for at least 4 h prior to CE analysis.

2.4 Production of dsDNA conjugates

Oligonucleotides used as PCR primers were purchased from Integrated DNA Technologies, and are shown schematically in Table 2. The oligonucleotides consist of an M13 forward primer with a 5'-thiol linker and an internal fluorescein-dT base, and a set of M13 reverse primers, with or without 5'-thiol linkers, designed to produce dsDNA products of 75, 100, 150, or 200 bp in size when used in a PCR reaction with the forward M13 primer.

PCR reactions were performed using Pfu Turbo polymerase (Stratagene, La Jolla, CA, USA). Eight reactions were carried out with 20 pmol of the fluorescently labeled, thiolated M13 forward primer, and 20 pmol of each of the M13 reverse primers shown in Table 2, in a total volume of 20 μL. M13mp18 control DNA from a sequencing kit (0.2 μL) (Amersham Biosciences, Piscataway, NJ, USA) was used as a template. The M13 template was PCR-amplified with 32 cycles of denaturation at 94°C for 30 s, followed by annealing at 54°C for 30 s and extension at 72°C for 60 s. Products were analyzed by 2.5% agarose gel electrophoresis to confirm the sizes of the dsDNA amplicons, and the products were stored at –20°C until subsequent use.

Thiolated PCR products were reduced using a large excess of TCEP. To do this, 7 μL of PCR product was mixed with 0.7 μL of 1 M TCEP (in 1 M Tris buffer), plus an additional 0.35 μL of 1 M Tris, resulting in a solution of pH ~ 5. This mixture was incubated for 2–2.5 h at 40°C. Excess TCEP as well as PCR reaction components was removed using QIAquick PCR purification spin columns (QIAgen, Valencia, CA, USA) according to the manufacturer's instructions, with elution of the purified DNA in 30 μL of 100 mM sodium phosphate buffer, pH 7.2.

Table 2. Oligonucleotides used as PCR primers for producing dsDNA conjugates with drag-tags at one or both ends.

Oligonucleotide	Sequence
M13-Forward	X_1 CCX_2TTTAGGG TTTTCCCAGT CACGACGTTG
75-Reverse	GAGTCGACCT GCAGGCATGC
75-Reverse-T	X_1 GAGTCGACCT GCAGGCATGC
100-Reverse	GAGCTCGGTA CCCGGGGATC
100-Reverse-T	X_1 GAGCTCGGTA CCCGGGGATC
150-Reverse	GCGGATAACA ATTTCACACA
150-Reverse-T	X_1 GCGGATAACA ATTTCACACA
200-Reverse	CCAGGCTTTA CACTTTATGC
200-Reverse-T	X_1 CCAGGCTTTA CACTTTATGC

X_1, 5'-thiol linker with 6-carbon spacer; X_2, internal fluorescein-dT base

The purified PCR products (with one or two reduced thiols, depending on the reverse primers used) were split into multiple aliquots, and treated with one of four maleimide-activated drag-tags: NMEG-44, branched NMEG-70, P1–169, or P2–127. The amounts of drag-tag were sufficient in most cases to produce significant quantities of DNA with one or two drag-tags. Additional aliquots were treated with excess maleimide, to simply cap the reduced thiols and prevent further reaction or dimerization.

2.5 CE analysis of conjugates

Free-solution CE analysis was performed using an Applied Biosystems Prism 3100 Genetic Analyzer (Applied Biosystems), using an array of 16 fused-silica capillaries with inner diameter of 50 μm and a total length of 47 cm (36 cm to the detector). The running buffer was 89 mM Tris, 89 mM TAPS, 2 mM EDTA, pH 8.5, and 1% v/v POP-6 polymer solution to act as a wall-coating agent, with the adsorbed poly(dimethylacrylamide) effectively suppressing the EOF [32]. (The resulting polymer concentration is very low, and does not lead to any size-based sieving of the DNA.) Samples were diluted in water prior to analysis, to provide signals of appropriate strength for the fluorescence detector. The ssDNA samples were analyzed at 55°C, whereas dsDNA samples were analyzed at 25°C to prevent denaturation. Samples were introduced into the capillaries by electrokinetic injection at 1 kV (22 V/cm) for 2–20 s. Separations were carried out at 15 kV (320 V/cm). The fluorescein label of the DNA was detected in the "G" channel of ABI Dye Set E5, with λ_{max} 530 nm.

3 Results

3.1 Analysis of ssDNA conjugates

The experimental protocol in which ssDNA was mixed with different amounts of maleimide-activated drag-tag allowed the successful production of species with zero, one, or two drag-tags, which were easily separated and identified by free-solution CE analysis. This is illustrated in Fig. 2 for the case of the T40-dithiol DNA with NMEG-40 drag-tags. As seen in Fig. 2A, DNA with no drag-tag

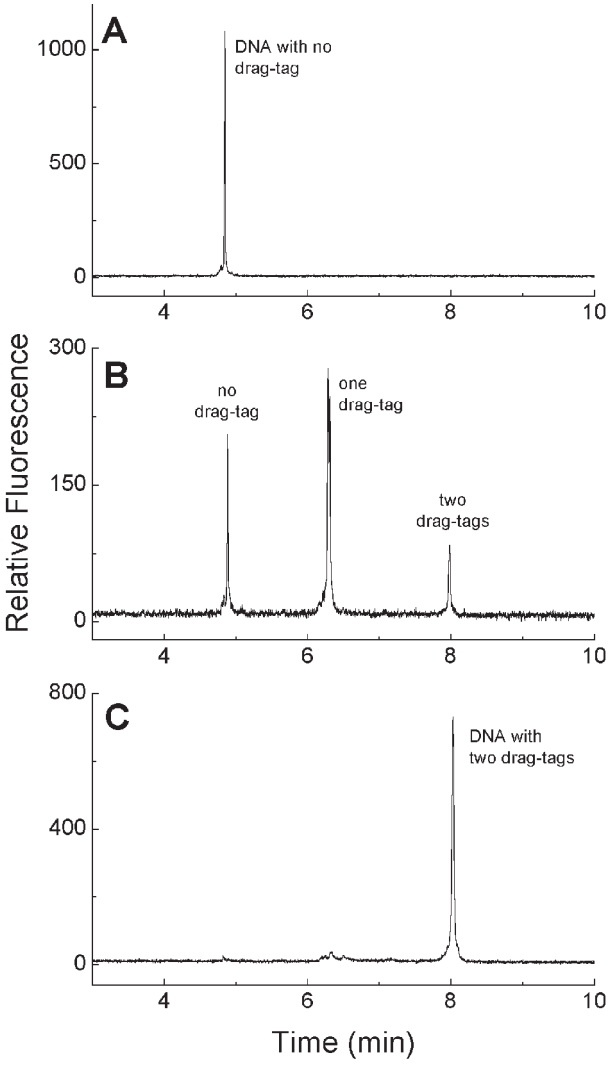


Figure 2. T40-dithiol DNA (A) capped at both ends with excess maleimide to create unlabeled ssDNA, (B) mixed with a 15:1 molar excess of NMEG-40 drag-tag followed by excess maleimide to create a mixture of unlabeled ssDNA and ssDNA with one or two drag-tags, and (C) mixed with a 100:1 molar excess of NMEG-40 drag-tag to create doubly labeled ssDNA. Samples were analyzed on an ABI 3100 capillary array instrument in 47 cm capillaries (36 cm to detector) in 89 mM Tris, 89 mM TAPS, 2 mM EDTA buffer, pH 8.5, with 1% v/v POP-6 polymer as a dynamic coating. Samples were injected electrokinetically at 22 V/cm for 3 s (A) or 2 s (B and C), and run at a field strength of 320 V/cm, with a current of 15 μA *per* capillary.

migrated as a single sharp peak with an electrophoretic mobility $\mu_0 = 3.9 \times 10^{-4}$ cm^2/V·s. Adding a 5- to 20-fold molar excess of the drag-tag to the DNA resulted in mixtures containing significant amounts of DNA with zero, one, or two drag-tags, as shown in Fig. 2B. Adding the drag-tag in a much larger molar excess (100-fold, relative to the DNA) led to nearly complete reaction of both ends of the DNA, again resulting in a single sharp peak as seen in Fig. 2C. Residual TCEP, present at 40-fold excess during the reduction, interferes somewhat with the reaction of the free thiols with the maleimide-activated drag-tags, and it was found that a significantly greater than 40-fold molar excess of drag-tag was necessary to achieve complete derivatization of both ends of the DNA. Species that were identified as ssDNA with one drag-tag typically appeared as a doublet of closely spaced peaks, as with the middle peak in Fig. 2B. The reason for this was not immediately obvious, but one possibility is that slight differences in electrophoretic mobility arise from labeling at the 5'- end or 3'- end of the DNA molecule, since the thiol linkers at the two ends are of different lengths.

In the optimized protocol, excess maleimide was used to cap any remaining unreacted thiols. We did this because, in initial attempts to produce mixtures comprising significant amounts of DNA with zero or one drag-tag, additional peaks would appear at characteristic spots in the electropherogram, particularly between the peaks for DNA with one and two drag-tags, and trailing the peak for DNA with two drag-tags. The extra peaks would be absent when the samples were first analyzed, but would grow in magnitude over the course of hours to days after the reduction of the DNA and reaction with the drag-tags. Although the extra peaks were never conclusively identified, it was hypothesized that they resulted from reoxidation of some of the residual free thiols to form disulfides. The addition of excess maleimide about 2 h after the addition of the drag-tag effectively prevented this problem, as the maleimide rapidly reacts with any remaining free thiols. The capping of both ends of the dithiolated DNA with this small molecule was found to induce a small, almost negligible mobility shift of 2–3 s relative to reduced, uncapped dithiolated DNA (data not shown), corresponding to an additional drag for the maleimide moiety equivalent to ~0.1 bases of DNA, as calculated in Section 4.

For each drag-tag (NMEG-20 or NMEG-40), samples consisting of both sizes of DNA (T20-dithiol or T40-dithiol) with zero, one, or two drag-tags were pooled to create mixtures containing multiple species, which were then separated and analyzed by CE. Run-to-run and capillary-to-capillary variabilities in migration time were generally quite low (approximately ± 1%), allowing easy identification of peaks in the pooled samples by comparing to the migration times of the individual components prior to pooling. CE analyses of these pooled mixtures are shown in Fig. 3, along with the peak assignments. A simple visual inspection confirms the general predictions of the end-

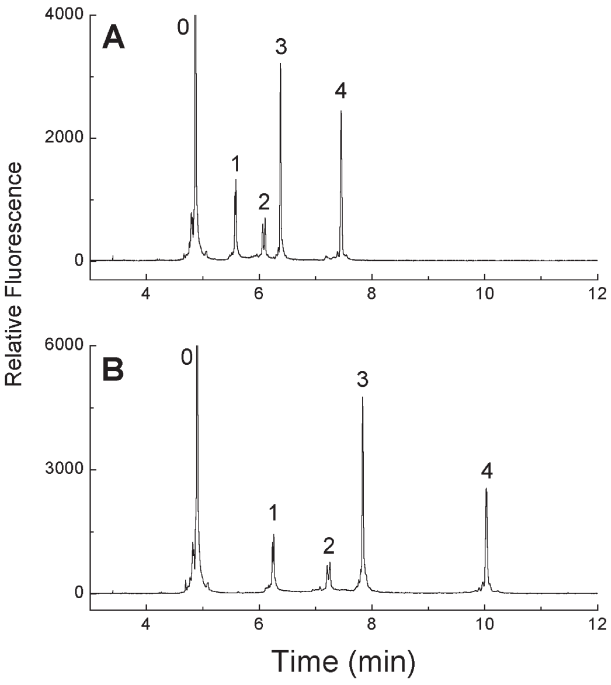


Figure 3. CE analysis of mixtures of 20mer and 40mer DNA with (A) NMEG-20 drag-tag, and (B) NMEG-40 drag-tag. Analysis conditions are the same as Fig. 2, except the injection was 22 V/cm for 15 s. Running current was 15 μA *per* capillary. Peak assignments for both (A) and (B) are: 0 = maleimide-capped DNA (no drag-tag); 1 = 40mer DNA with one drag-tag; 2 = 20mer DNA with one drag-tag; 3 = 40mer DNA with two drag-tags; 4 = 20mer DNA with two drag-tags.

effects theory: 20mer DNA with two 20mer drag-tags (Fig. 3A, Peak 4) migrates more slowly than 20mer DNA with one 40mer drag-tag (Fig. 3B, Peak 2), and likewise for the 40mer DNA (compare Fig. 3A, Peak 3 and Fig. 3B, Peak 1).

The apparent overall frictional parameter $\alpha = \alpha_1 M_U$ (as given by Eq. 1) could be computed directly from the peak times in Fig. 3. The α value calculated through use of Eq. (1), which neglects the end-effect, is termed the "apparent" α value so as to distinguish it from that determined using Eqs. (3) and (5), which account for the end-effect, as will be discussed in Section 4. The apparent α values, which qualitatively display the trend expected from the end-effects theory, are shown in Table 3. It is evident that two drag-tags give more than double the drag of a single tag, with roughly 6–9% enhancement for two drag-tags on ssDNA *versus* the expected drag for a single tag of twice the size. These experimental results will be analyzed quantitatively in Section 4, using the more detailed theory taking end-effects into account.

Table 3. Apparent frictional parameter α for ssDNA with one or two drag-tags calculated from peak times in Fig. 3, with correction made for the slight mobility shift arising from the maleimide capping

DNA length	Drag-tag	Apparent α	Error ($\pm$)	Ratio ($\alpha_{(2)}/2\alpha_{(1)}$)
20	NMEG-20 (one)	5.1	0.07	1.07
	NMEG-20 (two)	10.9	0.1	
20	NMEG-40 (one)	9.7	0.1	1.09
	NMEG-40 (two)	21.2	0.2	
40	NMEG-20 (one)	6.1	0.08	1.06
	NMEG-20 (two)	12.9	0.2	
40	NMEG-40 (one)	11.2	0.2	1.09
	NMEG-40 (two)	24.5	0.3	

Final column gives the ratio of the drag for a tag at each end *vs.* the expected drag for a single tag of twice the size. Error margins on experimentally determined α values assume an uncertainty of $\pm$0.05 min in peak times, which reflects the run-to-run and capillary-to-capillary variability observed with the instrument.

It is also clear from the results in Table 3 that the apparent α for a given size of drag-tag depends on the size of the DNA. For example, two NMEG-20 drag-tags on the 20mer DNA give α = 10.9, whereas the same two NMEG-20 drag-tags on the 40mer DNA give α = 12.9 – a difference of 18%. This is in agreement with the end-effects theory: For a drag-tag of a fixed size on one or both ends, a longer DNA molecule means that the drag-tag monomers are relatively closer to the chain end (*n*/*N* closer to 0 and/or 1), thereby giving the drag-tag monomers a heavier weighting in determining the mobility of the conjugate. Thus, the apparent α value for a given drag-tag on one or both ends of the DNA increases as the DNA chain length increases.

3.2 Analysis of dsDNA conjugates

dsDNA conjugate molecules were produced by performing PCR using a thiolated forward primer and normal (unthiolated) reverse primer (for production of dsDNA conjugates with a drag-tag at one end only), or using thiolated forward and reverse primers (for production of dsDNA conjugates with drag-tags at both ends). A large excess of TCEP was used for reduction of the thiols after the PCR reaction. Since TCEP is supplied as an HCl salt, the use of a large excess results in an acidification of the PCR buffer. To compensate for this, and to prevent long-term exposure of the DNA to very acidic conditions, additional 1 M Tris was added to the reduction mixture,

resulting in a more acceptable pH. Following the reduction, the PCR products were purified using QIAquick spin columns, which effectively remove residual buffer salts, surfactants, enzyme, and reducing agents left over from the PCR reaction and reduction, which might otherwise interfere with reaction with the drag-tags.

The drag-tags used for the dsDNA conjugates were two moderately large synthetic polypeptoids (linear NMEG-44 and branched NMEG-70), and two protein polymers produced by genetic engineering of *Escherichia coli*. The branched NMEG-70 and the P1–169 drag-tags have been described previously for the separation of denatured (single-stranded) PCR products of sizes similar to those described here [22, 31]. In this study, CE analysis was performed at room temperature with no denaturants in the buffer, ensuring that the DNA remained in its double-stranded state. Keeping the DNA in its double-stranded state allows for the easy incorporation of a drag-tag at both ends, which was expected to generate more than twice the drag of a single drag-tag, allowing the separation of a wider size range of dsDNA molecules.

The concentration of the DNA purified with the QIAgen spin column was too low for accurate measurement of absorbance at 260 nm, and thus the molar ratios of DNA to drag-tag are not known precisely. The amounts of drag-tag were generally sufficient to produce significant amounts of product with zero and one drag-tag (for products with only the forward primer thiolated), and zero, one, and two drag-tags (for PCR products with both primers thiolated). Typical electropherograms for two sizes of DNA (100 bp and 200 bp) with the P2–127 protein polymer are shown in Fig. 4. In each case, the migration time of the "free" DNA (with no drag-tag) is around 6.2 min. In panels (A) and (C), which show PCR products generated with only a thiolated forward primer, the free DNA peak is followed by a single peak, corresponding to DNA with a single drag-tag. In panels (B) and (D), which show PCR products generated with both forward and reverse thiolated primers, there is an additional peak 1–2 min later, corresponding to DNA with a drag-tag at both ends. Note also in panels (B) and (D) that, for the products generated with both primers thiolated, there are two closely spaced peaks migrating around the same time as the product with one drag-tag in panels (A) and (C). As with the split peaks for the ssDNA conjugates with one drag-tag, the exact cause of this phenomenon is unknown, but it was observed for all sizes of dsDNA with all of the drag-tags, and may result from slight differences in electrophoretic mobility arising from labeling at either end of the DNA molecules.

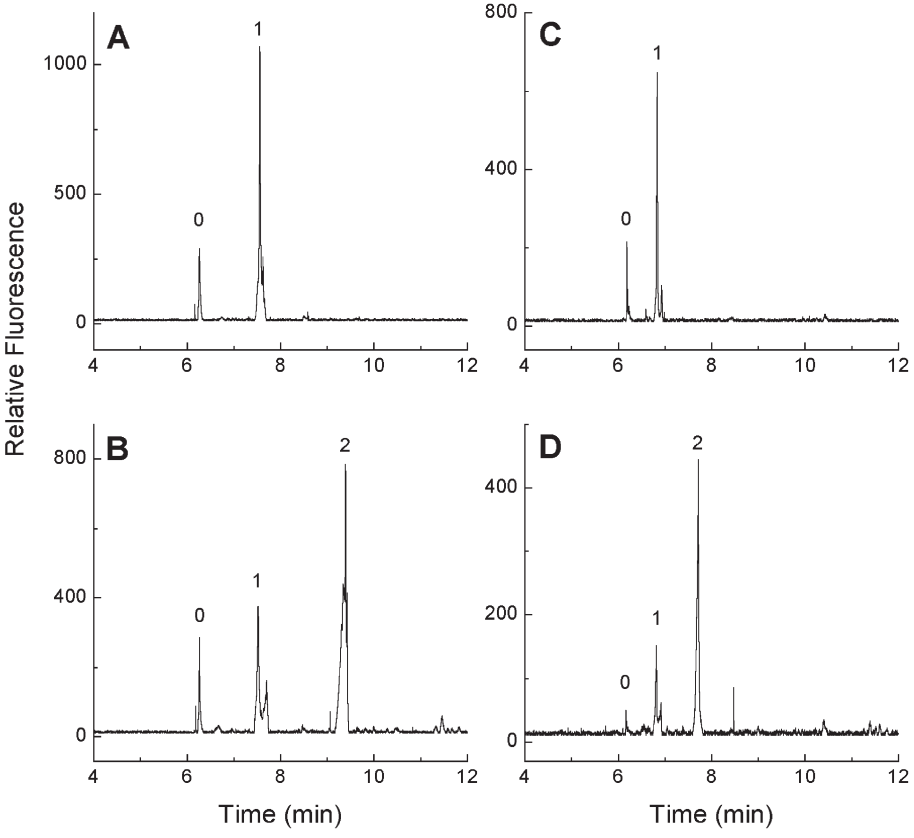


Figure 4. Electropherograms of dsDNA conjugated to P2–127 drag-tag. (A) 100-bp PCR product with forward primer thiolated, (B) 100-bp PCR product with both primers thiolated, (C) 200-bp PCR product with forward primer thiolated, and (C) 200-bp PCR product with both primers thiolated. Analysis conditions were the same as Fig. 2, except the run temperature was 25°C and the injection was 1 kV for 20 s. Peaks labeled 0, 1, and 2 refer to DNA species with zero, one, or two drag-tags, respectively.

The P1–169 and P2–127 protein polymers used here as drag-tags were not entirely monodisperse [22], leading to some additional peak broadness. The additional broadness is most noticeable with the smaller sizes of DNA, and is more pronounced for the species with two drag-tags. Both of these effects are as expected. Sharper peaks for larger sizes of DNA conjugated to impure drag-tags (including P1–169) were reported in [22], and are also in line with theory presented in [33]. The conjugation of a polydisperse drag-tag to both ends of a DNA molecule leads to a large number of possible combinations, each with slightly different electrophoretic mobility, which is apparent as additional peak broadness. The NMEG-44 and branched NMEG-70 drag-tags, both of which were purified to near monodispersity by RP-HPLC, generate cleaner, sharper peaks than the protein polymer drag-tags (data not shown).

Alpha values were calculated from the peak migration times of each species. In previous ELFSE literature, the relative mobilities of unlabeled and labeled DNA (μ_0/μ) would be plotted with respect to $1/M_C$, resulting in a straight line with slope α [18, 19]. This approach neglects the end-effects theory, which predicts a different overall value of α for each size of DNA. In this case, such plots are still essentially linear (not shown), and can be used to give an average apparent value of α for each drag-tag, as given in Table 4. (Note that the average α values determined by the linear fit of μ_0/μ *vs.* $1/M_C$ are not necessarily equal to the arithmetic average of the individual α values calculated for each size of DNA.) As indicated by the right-most ("Ratio") column in Table 4, the average α for two drag-tags is noticeably greater (10–23%) than twice α for a single-drag-tag, for these dsDNA species.

Table 4. Apparent frictional parameter α for dsDNA with one or two drag-tags, averaged for all sizes of DNA

Drag-tag	Average α	Ratio $(\alpha_{(2)}/2\alpha_{(1)})$
NMEG-44 (one)	12.7	1.10
NMEG-44 (two)	28.0	
Branched NMEG-70 (one)	17.0	1.22
Branched NMEG-70 (two)	41.6	
P1–169 (one)	27.2	1.13
P1–169 (two)	61.7	
P2–127 (one)	19.9	1.23
P2–127 (two)	48.8	

Final column gives the ratio of the drag for a tag at each end *vs.* the expected drag for a single tag of twice the size.

4 Discussion

The results we obtained for the analysis of ssDNA conjugates with poly(NMEG) drag-tags can be compared directly to the predictions from the end-effect theory presented in Eqs. (3) and (5). To take the end-effect into account, the weighting function presented in Eq. (4) is used. The parameter α_1 for scaling the uncharged monomers can be calculated using the end-effect theory, but we must first account for the slight additional drag arising from the maleimide moiety added to cap any unreacted thiols. To find the drag α_m associated with a single maleimide cap, the following equation was solved (using Maple):

$$t = \frac{t_0(M_c + 2\alpha_m)}{\int_{\alpha_m}^{\alpha_m + M_c} \Psi\left(\frac{n}{M_c + 2\alpha_m}\right) dn} \quad (6)$$

where t_0 is the arrival time of the uncapped DNA, and t is the arrival time of the DNA capped on each end with maleimide. For the 20-base DNA, α_m was found to be 0.035, while for the 40-base DNA it was found to be 0.052. Since the end-effect theory was derived for long Gaussian chains, it is assumed that the α_m value found for the larger DNA chain more closely represents the true value.

Note that the fluorescein-dT base near the middle of the chain likely exerts some effect on the mobility, as the fluorescein carries a -2 charge, and the dye along with the spacer arm linking it to the dT base likely add some hydrodynamic friction. To properly account for this effect would require a dithiolated oligonucleotide with no fluorescein, which would be undetectable with the CE instrument used for the analysis. The effect of the fluorescein is likely moderated by its position near the middle of the DNA chain (and hence its lower weight in determining the electrophoretic mobility). Additionally, the experimental determinations of α were made by comparing mobilities of drag-tag-labeled and free DNA, all of which were labeled identically with fluorescein. The impact on the results is expected to be minimal, and thus the contributions of the fluorescein as well as the thiol linkers present on all of the DNA species are ignored.

For DNA with one drag-tag and one maleimide cap, α_1 for the drag-tag can be found by solving Eq. (7)

$$t_1 = \frac{t_0(M_c + \alpha_m + \alpha_1 M_u)}{\int_{\alpha_m}^{\alpha_m + M_c} \Psi\left(\frac{n}{M_c + \alpha_m + \alpha_1 M_u}\right) dn} \quad (7)$$

where t_0 is the arrival time of the DNA with no drag-tag (after correcting for the presence of maleimide caps on each end), and t_1 is the arrival time of the DNA with one maleimide cap and one drag-tag. The calculated values of α_1 are presented in Table 5. Note that the closely

Table 5. Values of α_1 for NMEG drag-tags calculated from experimental data for ssDNA, taking into account the theory of end-effects

DNA length (M_C)	Drag-tag length (M_U)	α_1
20	20	0.19
	40	0.21
40	20	0.20
	40	0.21

spaced doublet for the arrival time of these singly labeled molecules was averaged for the results presented in Table 5; using either the faster or slower times resulted in α_1 values that differed from the average by a negligible amount. Note that the values of α_1 increase slightly with increasing size of the conjugate. For a given class of polymer, α_1 is expected to be a constant that is related to the chemical structures of the components and the experimental conditions (*i.e.* monomer size and Kuhn length, ionic strength of the buffer). The slight variation among the conjugates is likely due to the fact that the DNA and the drag-tags are too small to be perfectly Gaussian in conformation, which is an underlying assumption for the theory of ELFSE. Since the largest molecules are expected to be the closest to being Gaussian in conformation, we use the corresponding value of $\alpha_1 = 0.21$ to represent the true value for the poly(NMEG) drag-tags under the current experimental conditions.

Using the end-effect theory, the predicted arrival time for DNA with two drag-tags is

$$t_2 = \frac{t_0(M_c + 2\alpha_1 M_u)}{\int_{\alpha_1 M_u}^{\alpha_1 M_u + M_c} \Psi\left(\frac{n}{M_c + 2\alpha_1 M_u}\right) dn} \quad (8)$$

Equations (7) and (8) can now be used to predict the ratio of the mobilities of a bioconjugate with two drag-tags to the mobility of a conjugate with one drag-tag of twice the size, $\mu_2/\mu_1 = t_1/t_2$. The values predicted from Eqs. (7) and (8), using $\alpha_1 = 0.21$, are given in Table 6, along with the experimentally observed values, for the cases of 20mer or 40mer DNA with either a single 40mer drag-tag, or two 20mer drag-tags. The experimental results are closer to the value of 1, which is that predicted by the simple theory in Eq. (1) that neglects end-effects. The experimental value for the 40mer DNA is closer to the values predicted by the end-effect theory; this may be because the larger chains more closely approximate Gaussian coils, and are thus more appropriate test cases for the theory.

The quantitative end-effect theory is not directly applicable to the dsDNA data presented here. Although the dsDNA products are significantly longer, dsDNA is also considerably stiffer, with a much longer persistence length than ssDNA. Thus, even the longer dsDNA products are more likely to resemble stiff rods or cylinders, rather than random coils. Even with such a geometry, there is still likely an end-effect, which is dramatically illustrated by the experimental measurements of α presented in Table 4. Since the dsDNA-drag-tag conjugates are not likely to even approximate Gaussian coils, application of the theory used for the ssDNA conjugates is not appropriate.

Table 6. Mobility ratio μ_2/μ_1 for two 20mer drag-tags (μ_2) *vs.* one 40mer drag-tag (μ_1)

DNA length (M_C)	Predicted μ_2/μ_1	Experimental μ_2/μ_1
20	1.08	1.03
40	1.05	1.03

The drag enhancement for placing a drag-tag at each end of dsDNA is noticeably larger than was observed for placing a drag-tag at each end of ssDNA. This could simply be a function of the specific sizes of DNA and drag-tags that were chosen for study, but it may also be the result of the stiff rod-like structure of the dsDNA. Because the dsDNA molecules studied here are relatively short, the ends of the dsDNA molecule are more often on the "outside" of the chain, as opposed to a true Gaussian coil for which the chain ends may occupy positions in the interior of the coil. In addition, there may be a greater degree of hydrodynamic segregation between the rod-like dsDNA and the random coil drag-tags. Detailed theoretical analysis is required to determine if these simple arguments can explain the larger end-effect observed for dsDNA in these experiments.

The enhanced drag arising from placing a drag-tag at both ends of DNA leads to interesting new possibilities for sequencing and genotyping by ELFSE. The separation capacity of ELFSE is tied directly to the amount of friction generated by the drag-tag, and previous efforts have been focused on creating larger drag-tags to generate more friction. The possibility of including a drag-tag at both ends extends the range of separations that are possible with existing drag-tags. This is particularly important as the production of very large, totally monodisperse protein polymer drag-tags has proven difficult [22, 29]. The direct application of this technique to DNA sequencing would be difficult with current commercially available dye terminator chemistry, which presents no convenient functional group for attaching a second drag-tag at the 3'-end of the sequencing fragment. The dithiolated ssDNA oligos used in this study were custom-synthesized at considerable expense, and further appli-

cation to separation of ssDNA will likely require new developments in sequencing chemistry. The application of labeling both ends to the separation of dsDNA generated by PCR is more straightforward, given the wide availability of custom-synthesized DNA primers with a variety of functional groups and linkers that can be incorporated at the 5'-end.

In conclusion, this study has provided verification of an important and interesting prediction of the new theory of end-effects in ELFSE separations. Using both custom-synthesized ssDNA oligonucleotides and larger dsDNA products generated by PCR, labeled at one or both ends with a variety of drag-tags, it has been shown that the drag induced by labeling both ends is more than double the drag arising from a single drag-tag at one end, and is also larger than the drag that would arise from a single drag-tag of twice the size at one end. The effect is significant, with drag (α) enhanced by 6–9% for the ssDNA and by 10–23% for the dsDNA in the size range tested with the available drag-tags. This enhanced drag from double end-labeling could potentially be useful for various types of ELFSE separations such as DNA sequencing, if a suitable experimental approach can be developed for incorporating a drag-tag on each end of the DNA prior to analysis.

While the experimental data qualitatively show the trends expected from the end-effect, the data for ssDNA differ somewhat with the quantitative theoretical predictions. The agreement between the experimental data and the theory is closer for the larger molecules studied, which may be because these molecules are more Gaussian in conformation. Ideally, much larger (and hence more Gaussian) ssDNA and drag-tags could be used to test the end-effect theory; however, the direct synthesis of very long, doubly thiolated, and internally fluorescently labeled oligonucleotides, followed by purification to monodispersity, would be low-yielding and cost-prohibitive. Further tests with much larger ssDNA will require a different approach to creating long ssDNA with functional groups on each end appropriate for conjugation of a drag-tag.

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