INTRODUCTION

The development of novel and improved technologies for DNA separation and analysis continues to be driven by a societal need to make sequencing and genotyping more cost-effective. Capillary electrophoresis (CE)\(^1\) and capillary array electrophoresis (CAE) enable high-throughput DNA sequencing and genotyping separations by allowing the use of higher electric fields and greater automation than was possible with the traditional slab gel format. Both CE and CAE require the use of highly viscous polymer solutions (e.g., entangled solutions of linear polyacrylamide) as intracapillary DNA size-separation matrices. In addition to their expense, these polymer solutions are required to apply high pressure to be loaded into narrow capillaries, and generally DNA sequencing read lengths, with even the best polymers and CAE instruments, are limited to about 800 bases at best.

DNA separation cannot normally be achieved by electrophoresis in free solution, that is, in the absence of polymer networks, because the electrophoretic mobility of DNA molecules is independent of their chain length \((1)\). It was theorized in 1992 that DNA could be separated by free-solution electrophoresis if one attached a monodisperse perturbing entity or “drag-tag” to DNA fragments of varying size \((2)\). That is, it was predicted that the charge-to-friction ratio of DNA can be rendered size-dependent if a monodisperse drag-tag is covalently attached to one end of the DNA molecules to be separated, allowing the DNA chains to be separated by microchannel electrophoresis in a regular, size-dependent fashion. This approach, called end-labeled free-solution electrophoresis (ELFSE), has been under development for the past 10 years as a promising bioconjugate method of DNA sequencing and genotyping that could eliminate the need for viscous polymer solutions in capillary and chip electrophoresis of DNA \((3–5)\). Experimentally, ELFSE with various types of drag-tags has been used to separate short oligonucleotides with high resolution \((6, 7)\), as well as long double-stranded DNA fragments \((8)\). For DNA sequencing applications, ELFSE promises to provide faster separations and longer read lengths than matrix-based electrophoresis and should be particularly well suited for use in microfluidic devices \((5, 9, 10)\).

The amount of drag created by the drag-tag can be characterized in terms of the parameter “\(a\), which has the units of the hydrodynamic drag of a single base of...
ssDNA (4). In circumstances and conditions likely to be present in this study, recent theoretical treatments of the electrophoretic mobilities of composite molecules from the Slater group (11, 12) interpret $\alpha$ in terms of hydrodynamic “blobs”. The effective friction coefficient $\alpha$ for a drag-tag can be estimated experimentally by measuring (simultaneously) the electrophoretic migration times of unconjugated, “free” DNA and of a drag-tag—DNA conjugate comprising the same DNA. The parameter $\alpha$ can then be calculated with the equation

$$\alpha = N\left(\frac{M_0}{\mu} - 1\right)$$

for a conjugate molecule consisting of $N$ bases of DNA and a charge-neutral drag-tag with friction equivalent to $\alpha$ bases of ssDNA, having the electrophoretic mobility $\mu$, where $M_0$ is the electrophoretic mobility of unlabeled DNA (about $2.5 \times 10^{-4}$ cm²/(V-s) for the conditions herein) (4).

High-resolution ELFSE separations of DNA require the ideal drag-tag to be (i) totally monodisperse, (ii) of high enough molecular weight to impart sufficient drag to separate DNA analytes of varying size, (iii) watersoluble and polar but essentially charge-neutral (13), and (iv) resistant to nonspecific (band broadening) interactions with microchannel walls. Taken together, the various and in some cases contradictory design criteria make drag-tag design and synthesis a challenging problem in molecular engineering.

Natural proteins and viruses have been proposed as candidate drag-tags (2, 4). This prediction was validated by the successful sequencing of ca. 110 DNA bases in 18 min by free-solution CE with the use of the protein streptavidin as a drag-tag (14). This result, although remarkable, highlighted some significant drawbacks of using natural proteins as drag-tags. For instance, the results indicated a problem with obtaining streptavidin in a truly monodisperse preparation and also showed that even a virtually charge-neutral protein can suffer from strong adsorptive interactions with the microchannel wall. These interactions result in band broadening and a decrease in peak resolution. Streptavidin is a tetrameric protein (15) ($4 \times 13$ kDa, totaling 536 amino acid residues) that has numerous biochemical applications (16), but despite its relatively large size, it adopts a compact, globular conformation, resulting in a relatively low drag, equal to approximately 30 bases of DNA. This was the primary factor limiting read length in the study by Slater and Drouin (14).

Synthetic polymers have also been examined for use as ELFSE drag-tags. Poly(ethylene glycol) (PEG) is water-soluble, relatively nonadsorptive to glass, hydrophilic, and uncharged – all potentially ideal molecular properties for ELFSE. However, even PEGs with a low molecular mass such as 3400 g/mol (17) and an ultralow polydispersity index ($M_d/M_w$) of 1.01 are not sufficiently monodisperse for DNA sequencing or genotyping applications (18).

Poly-N-substituted glycines (polypeptoids) are nonnatural, sequence-specific polymers based on a polyglycine backbone (19). This class of molecules may be synthesized on solid phase in high yield using a “sub-monomer approach,” to include a myriad of different side-chain functionalities (20, 21). After cleavage from the solid phase, they can be purified to monodispersity by reversed-phase high-performance liquid chromatography (RP-HPLC).

Vreeland et al. successfully employed a family of linear, poly(N-methoxyethyl glycine) peptoids (poly(NMEG)s) with PEG-like side chains (Table 1) as drag-tags for the ELFSE separation of short DNA oligonucleotides. Poly- (NMEG) drag-tags ranging in size from 10 to 60 monomers in length were used to separate 20- and 21-base ssDNAs (6), as well as single-base extension (SBE) reaction products between 19 and 24 bases in length (22). The longest polypeptoids used in these studies were 60 monomers in length, and were obtained in only modest yields by the divergent solid-phase peptide synthesis techniques used.

Aiming to improve upon this work, we hypothesized that the grafting of “branch” molecules onto a “backbone” would be an efficient convergent strategy for the synthesis of monodisperse, high-molecular weight drag-tags that could allow the separation of larger DNA. To test this idea, we designed a 30-mer poly(NMEG) “backbone” (Scheme 1) with five evenly spaced $\epsilon$-amino sites to be grafted via a peptide bond-forming reaction with tetramer (2) and octamer (3) oligo(NMEG) peptoids possessing a terminal carboxylic acid (Scheme 2). The amino groups arrayed along the backbone of 1 could also be acetylated, yielding a set of three drag-tags with increasing branch length. We anticipated that the testing of these three different molecules as drag-tags for DNA separation by free-solution CE would provide valuable information regarding the relationship between chain architecture, molecular weight, and hydrodynamic drag ($\alpha$). Grafting reactions could in theory be performed while the backbone molecule (1) is still on the solid-phase resin; however, for the coupling methodology to be more widely applicable for various classes of backbone molecules, we chose a solution-phase grafting strategy. In this research article, we describe how unbranched (acetylated), tetramer-branched, and octamer-branched comblike poly- (NMEG) peptoid drag-tags were synthesized, characterized, and evaluated. These drag-tags were attached to both 20- and 30-base DNA primers, and free-solution CE was used to analyze the electrophoretic mobilities of the bioconjugate molecules. We were able to observe and quantify the $\alpha$ values for each of these drag-tags. We also demonstrate the use of one of these drag-tags for the efficient separation of differently sized DNA fragments up to 150 bases long by CE in free solution.

### Table 1. N-Substituted Glycine (Peptoid) Side Chains

<table>
<thead>
<tr>
<th>Designator</th>
<th>R - Side chain</th>
<th>(N)-substituted glycine oligomer, or peptoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMEG</td>
<td>(N)-(methoxyethyl) glycine</td>
<td></td>
</tr>
<tr>
<td>NARG</td>
<td>(N)-(butylamine) glycine</td>
<td></td>
</tr>
</tbody>
</table>

### EXPERIMENTAL SECTION

#### General Methods and Materials. The “sub-monomer” synthesis of the poly-N-substituted glycines or “polypeptoids” used in this work has been described previously (23). Scheme 1 depicts the protocol that was used to synthesize the polypeptoid molecules made for
**Scheme 1. The Sub-Monomer Solid-Phase Polypeptoid Synthesis Protocol**

- **Step 1:** Cleavage from resin (95:2.5:2.5 TFA/TIS/H2O) in DMF
  
- **Step 2:** RP-HPLC purification

**Scheme 2. Chemical Structures of the Tetramer (a) and Octamer (b) “Branches”**

- **a)**
  - **Compound 2**

- **b)**
  - **Compound 3**

> They are oligo(N-methoxyethyl glycine) (oligo(NMEL)) peptoids with reactive terminal glutamic acid residues.

> Structure is predominantly poly(N-methoxyethyl glycine) (poly(NMEL)) residues with five evenly spaced N-butylamine (NABG) monomers included within. The final structure of “backbone” is shown with amino attachment sites deprotected.

> This study. All reactions were carried out on an ABI 433A automated peptide synthesizer (Applied Biosystems, Foster City, CA). All reagents used were purchased from Aldrich (Milwaukee, WI), unless stated otherwise. The mass spectra were recorded by MALDI-TOF (Voyager Pro DE, Perceptive Biosystems, Framingham, MA) and ESI (Waters Micromass Quatro II, Milford, MA). For synthesis details, see Supporting Information.

**Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC).** Analytical RP-HPLC was performed on a column with C18 packing (Vydac, 5 μm, 300 A, 2.1 mm × 250 mm). The following conditions were employed, unless otherwise stated: a linear gradient of 10–40% B in A was run over 50 min at a flow rate of 0.5 mL/min (solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in acetonitrile) at 58 °C; analytes were detected by UV absorbance at 220 or 260 nm or both. Preparative HPLC was performed on a Vydac C18 column (Vydac, 15 μm, 300 A, 22 mm × 250 mm) using the same solvent and detection systems; analytes were eluted with a linear gradient of 10–40% B in A over 50 min at 12 mL/min.

**Polypeptoid “Backbone” (Compound 1).** Synthesis Details. Fmoc-Rink amide resin (Nova Biochem, San Diego CA. 0.30 mmol scale) was deprotected by treatment with piperidine in dimethylformamide (DMF) (20% v/v; 2 × 7 mL) in two consecutive 15-min treatments. The oligomer chain was then assembled with alternating cycles of the bromoacetylation step and amine displacement of the alkyl bromide moiety. Bromoacetylation was achieved by mixing the resin with bromoacetic acid (BAA) (1.2 M, 4.3 mL) in DMF and disopropylcarbodiimide (DIC) (1 mL, 9.9 mmol). The mixture was vortexed for 45 min, the liquid was drained, and the resin was rinsed with DMF (4 × 7 mL). The resin was then mixed and vortexed (45 min) with either methoxymethylamine (1.0 M, 4 mL) or mono-Boc-protected diaminobutane (1.0 M, 4 mL) (24) in N-methylpyrrolidone (NMP) to introduce the methoxyethyl (NMEG) or butylamine (NABG) side chain moieties (Table 1). The liquid was drained, and the resin was rinsed with DMF (4 × 7 mL). These two reaction cycles were alternated until the polypeptoid was of the desired sequence and length. Finally, an Fmoc protecting group was installed on the amine terminus while the polypeptoid was still on the resin. This was achieved by adding Fmoc-glycine and DIC under the same conditions as used for the bromoacetylation step.

Finally, the polypeptoid was cleaved from the solid support by treatment with 95:2.5:2.5 trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water for 10 min. The polypeptoid was filtered through a fritted glass vessel to remove the solid support, diluted with water (50 mL), frozen (−80 °C), and then lyophilized. The product of the solid-phase synthesis was evaluated by analytical RP-HPLC. Preparative RP-HPLC was subsequently performed, and appropriate fractions were combined to afford the desired product (1) in pure preparation.

**Tetramer “Branch” (Compound 2).** Using the same methods described above, we synthesized this oligopeptoid using the “sub-monomer” approach (23) on a 0.30 mmol scale on Fmoc-Rink amide resin (Scheme 2a). Following procedures outlined above, four additions of the N-methoxyethyl glycine monomer were followed by the addition of an N-α-Fmoc-l-glutamic acid γ-tert-butyl ester (Fmoc-Glu(OctBu)-OH) residue, which involves the coupling of the terminal secondary amine. This coupling was achieved by dissolving the amino acid in NMP (1.0 M; 4 mL) with PyBroP (1.2 M) and DIEA (1.2 M). The Fmoc group at the N-terminus of the glutamic acid residue was subsequently removed using 20% v/v piperidine in DMF, and the resultant primary amine was capped with acetic anhydride. This was achieved by immersing the resin in fresh acetic anhydride (neat) (Applied Biosystems, Foster City, CA), followed by agitation with occasional venting for 15 min. The resin was filtered and cleaved, as previously described. For the branch molecules, cleavage from the resin also resulted in deprotection of the t-butyl-protected glutamic acid side chain functionality, revealing the carboxylic acid group. The oligopeptoid was cleaved from the solid support, frozen, and lyophilized, as previously described.
identified by ESI mass spectrometry and analyzed by RP-HPLC to afford the desired tetramer (1).

Octamer "Branch" (Compound 3). The octamer branch molecule (Scheme 2b) was synthesized by preloading the Rink amide resin with Fmoc-Glu(StBu)-OH as the first step using standard peptide chemistry. The Fmoc group on the glutamic acid residue was subsequently removed using (20% v/v) piperidine in DMF, followed by the addition of eight monomers of N-methoxethyl glycine. The terminal secondary amine was acetylated, and the oligopeptoid was then cleaved from the solid support, frozen, and lyophilized, as previously described. Preparative RP-HPLC was performed, and appropriate fractions were combined to afford compound 3. The product was identified by ESI mass spectrometry and was analyzed by RP-HPLC.

Backbone Acetylation (Compound 4). An “unbranched” 30-mer drag-tag was synthesized by the addition of neat acetic anhydride (1 mL) to the purified backbone molecule 1 (5.0 mg, 1.3 μmol) (Scheme 3). Excess acetic anhydride was removed in vacuo, and the reaction mixture was quenched with water (10 mL), cooled (∼80 °C), and lyophilized, as previously described. Preparative RP-HPLC was performed, and appropriate fractions were combined to afford compound 4. The product was identified by MALDI-TOF mass spectrometry and was analyzed by RP-HPLC.

Backbone Grafting Reactions (Compounds 5 and 6). Typical synthesis protocol. Tetramer 2 (25.5 mg, 39 μmol) and PyBroP (24.85 mg, 39 μmol) were added to a cooled solution (0 °C ice-bath) of backbone 1 (5.0 mg, 1.3 μmol) in NMP (1 mL) (Scheme 3). The resultant mixture was stirred under nitrogen for 5 min. The reaction mixture was stirred vigorously and diisopropylethylamine (DIEA) (5 mg, 39 μmol) was added dropwise via cannula under a positive nitrogen atmosphere. The reaction mixture was allowed to warm to room temperature, and the reaction was allowed to proceed for 3 h. NMP was then removed in vacuo, and the solution was diluted with deionized water (12 mL), frozen, and lyophilized. The grafting reaction was monitored by analytical RP-HPLC using a slightly different method than previously stated: A linear gradient of 10–60% B in A was run over 50 min at a flow rate of 0.5 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) at 58 °C on a C18 packing column (Vydac, 5 μm, 300 Å, 2.1 mm × 250 mm); analytes were detected by UV absorbance at 220 and 260 nm. Preparative HPLC was performed on a Vydac C18 column (Vydac, 15 μm, 300 Å, 22 mm × 250 mm) using the same solvent and detection systems; analytes were eluted with a linear gradient of 10–60% B in A over 50 min at 12 mL/min. Preparative RP-HPLC was performed, and appropriate fractions were combined. Compound 5 was then treated with 20% v/v piperidine in methanol (1 mL) to remove the terminal Fmoc protecting group and stirred for 20 min. The solvent was then removed in vacuo, and the resulting material was repurified by RP-HPLC as previously described. The same conditions were used, and similar results were obtained for the grafting reaction with the octamer branch to produce compound 6.

Sulfo-SMCC Conjugation. The drag-tags (4-6), displaying a free amine at the N-terminus, were dissolved in 0.1 M sodium phosphate buffer, 0.15 M sodium chloride, pH = 7.18. Sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) (Pierce Scientific, Rockford, IL, 10 mg/mL in water) was added to the drag-tag/buffer solution. This reaction mixture was agitated at room temperature for 1 h. The reaction was quenched by the addition of water (2 mL) and then purified by preparative HPLC using the conditions described above. The appropriate fractions were then combined.

Drag-tag–DNA Conjugation. Sulfo-SMCC-activated polypeptide drag-tags were individually conjugated to 5′-thiolated, fluorescently labeled DNA oligonucleotides of lengths 20 and 30 bases (Integrated DNA Technologies, Coralville, IA). The 20- and 30-base DNA sequences were 5′-X1GTGTGGTCCAGTCACGACGTT-3′ and 5′-X2CCX3- TTAGGTTTCCAGTCAGCCGTTG-3′, where X1 was a 5′ thiol modifier (C6 disulfide) and X3 was an internal fluorescein-labeled dT base. Both sequences are thiol modifier (C6 disulfide) and X3 was an internal fluorescein-labeled dT base. Both sequences are 25 pmol of thiolyser with DNA with 5000 pmol of tris(2-carboxyethylphosphine) hydrochloride (TCEP) (Acros Organics, Morris Plains, NJ) in a total volume of 10 μL.
Table 2. Oligonucleotide Sequences Used as Primers for PCR Products of 50, 75, 100, and 150 bp

<table>
<thead>
<tr>
<th>primer</th>
<th>DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>5′-X₁CCX₂TTTAGGGTTTCCCCAGTCACGAGTTG-3′s</td>
</tr>
<tr>
<td>Reverse Primers</td>
<td></td>
</tr>
<tr>
<td>M13-50</td>
<td>5′-TGGCAGCTGCGCTGGTATTA-3′</td>
</tr>
<tr>
<td>M13-75</td>
<td>5′-GAGCTCGACCTGAGCGATC-3′</td>
</tr>
<tr>
<td>M13-100</td>
<td>5′-GGCTTGATACCCGGGAGT-3′</td>
</tr>
<tr>
<td>M13-150</td>
<td>5′-GCGGATACAAATTTCCACA-3′</td>
</tr>
</tbody>
</table>

X₁ refers to a 5′ thiol linker (C6 disulfide), and X₂ refers to an internal fluorescein-dT base.

of sodium phosphate buffer (100 mM, pH 7.2) at 40 °C for 110 min.

Reduced DNA was conjugated to sulfo-SMCC-activated polypeptides by mixing 500 pmol of polypeptide with 12.5 pmol of the reduced DNA mixture (containing 500 pmol of TCEP) in a volume of 5–7 µL of sodium phosphate buffer (100 mM, pH 7.2). Conjugation reactions proceeded in the dark at room temperature for approximately 6 h before CE analysis.

Preparation of PCR Products. The 30-base thiolated, fluorescently labeled M13 oligonucleotide described above [5′-X₁CCX₂TTTAGGGTTTCCCCAGTCACGACGTTG-3′] was used as a forward primer. Four different 20-base oligonucleotides (Table 2) were used as reverse primers for generating PCR products of 50, 75, 100, and 150 bp in length. M13mp18 ssDNA obtained from Amersham Biosciences (Piscataway, NJ) was used as a template. PCR reactions using *Thermus aquaticus* (Tag) DNA polymerase were performed using an MJ Research DNA thermal cycler with 30 cycles of 95 °C for 1 min (denaturing), 55 °C for 1 min (annealing), and 72 °C for 2 min (elongation). The size of each PCR product (dsDNA) was confirmed via 3% agarose gel electrophoresis. The PCR products (with no additional purification) were reduced with Tris(2-carboxyethyl)phosphine (TCEP) and conjugated to drag-tags as described above for the DNA primers.

Free-Solution Capillary Electrophoresis. DNA–polypeptide conjugates were analyzed by capillary electrophoresis with laser-induced fluorescence detection, using either a BioRad BioFocus 3000 single-capillary instrument (BioRad, Hercules, CA) or an Applied Biosystems Prism 3100 genetic analyzer with an array of fused silica capillaries of length 36 cm and inner diameter 50 µm. Analysis conditions were identical to those described above for the BioFocus experiments, except the electric field was 320 V/cm, and samples were introduced by an electrokinetic injection of 22 V/cm for 2 s. The PCR product–polypeptide conjugates were diluted in deionized formamide (Applied Biosystems, Foster City, CA) and denatured at 95 °C for 5 min prior to electrophoretic analysis to yield single-stranded DNA–drag-tag conjugates.

RESULTS AND DISCUSSION

Branched Drag-Tag Design and Synthesis. Attaching large molecules onto a scaffold containing multiple grafting sites, and achieving full coverage, is a challenging proposition (25). For ELFSE applications, the grafting linkages need to be stable enough to withstand further conjugation reactions, purification steps, wide pH ranges, and the high temperatures used in thermal cycling. For this reason, we decided on peptide-bond linkages. We designed the backbone (1) to have regularly spaced, pendant ε-amino groups as attachment sites for tetramer (2) and octamer (3) branch molecules with terminal carboxylic acid groups. This is an attractive strategy as it allows for the convergent reaction between large molecules that have been purified to monodispersity. Although the resolving power of RP-HPLC could potentially allow for a subsequent removal of the incompletely grafted side-products, complete grafting is a desired outcome, because even slight impurities in the drag-tag are exposed by CE analysis and will limit its usefulness.

Indeed, early attempts in our lab to graft polyamide oligomers onto lysine-containing polypeptides resulted in a ladder of partially grafted products that ultimately proved too difficult to purify to monodispersity (26). In the present work, we have used poly(N-methoxyethyl glycine)s and their derivatives for both the backbone and branch structures and were able to produce highly monodisperse components in high yield. The structure of poly(NMEG) is consistent with recently proposed rules for creating chemical surfaces that are resistant to adsorption of proteins from solution. Specifically, it was found that surfaces presenting groups that are polar but uncharged and hydrophilic and contain hydrogen-bond acceptors (but not hydrogen-bond donors) are inert to adsorption of proteins from solution (27). We hypothesized that poly(NMEG), which has all of these structural features, would be resistant to adsorption from solution onto surfaces. This is a critical feature for capillary electrophoresis, where wall–analyte interactions are a major source of band broadening. Indeed, in previous ELFSE separations using streptavidin as a drag-tag, the interaction between streptavidin and the capillary wall was the primary factor limiting separation efficiency at high electric field strengths (14).

We had to design the drag-tag for two stages of conjugation: a grafting reaction that introduced the monodisperse branches and then a conjugation to DNA. To achieve this, the backbone molecule (1) was designed...
with a glycine group at the N-terminus (Scheme 1), and this resulted in a terminal amine protected with 9-fluorenylmethoxycarbonyl (Fmoc). The Fmoc protecting group is orthogonal to the tert-butyloxycarbonyl (Boc)-protected N-butylamine (NABG) ε-amino groups along the backbone and, when removed (after grafting), exposes a free N-terminal amine for DNA conjugation. Cleavage from the resin using trifluoroacetic acid (TFA) also conveniently cleaves the Boc groups on the side chains without affecting the Fmoc-protected amino terminus.

The terminal Fmoc-glycine residue also facilitates purification. The increased hydrophobicity afforded by the inclusion of the Fmoc group dramatically increases the HPLC retention time, thus helping separate the desired product from impurities. Also, the UV absorbance of the Fmoc moiety allows for facile identification of the product peak at 260 nm by RP-HPLC. The Fmoc amino group can then be easily deprotected at a later point for conjugation to DNA prior to ELFSE analysis.

**Backbone Synthesis.** The backbone (1) was synthesized (Scheme 1) using commercially available materials and mono-Boc-protected diaminobutane, the latter synthesized and purified in-house according to literature procedures. RP-HPLC analysis revealed a single major product peak, approximately 77% by area (Table 3) (greater than 99% coupling efficiency per monomer residue). Compound 1 was obtained in pure form by preparative RP-HPLC, and its correct mass was confirmed by ESI mass spectrometry.

**Branch Syntheses.** The tetramer and octamer branches were both synthesized in excellent yield (Table 3) and purified to monodispersity by RP-HPLC. They were designed as oligo(N-methoxyethyl glycine) peptoids with terminal glutamic acid (peptide) functionalities. Previous unsuccessful attempts in our lab to make similar tetramer and octamer branches suitable for high-yield grafting involved using a terminal glycine unit to provide the reactive carboxylic acid group. Specifically, we had synthesized tetramer and octamer oligo(N-methoxyethyl glycine) peptoids on preloaded glycine Wang resin that occurred on a secondary amine. For both branch molecules, however, synthetic yields were high: RP-HPLC analysis revealed a single major product peak; yields were approximately 95% by area for both designs (Table 3). In addition, highly efficient grafting was achieved with both designs as described below (Figure 1).

**Grafting of Oligomeric Branches to the Polymeric Backbone.** The “unbranched” or N-acetylated drag-tag (4) was synthesized by the addition of acetic anhydride to a solution of the backbone (1) in methanol. Removal of the solvents in vacuo, followed by RP-HPLC purification, afforded a single pure product. The Fmoc protecting group was then removed from the isolated product using piperidine; analysis of the products of this deprotection reaction gave a single peak by RP-HPLC. Compound 4 was collected as a white solid.

One of the most challenging aspects of this synthesis was to achieve complete reaction at all five amino sites on the backbone molecule with the tetramer and octamer branches. To generate a completely monodisperse product, we had to find the best way to couple several large molecules together while forming bonds that are extremely stable. Peptide bond linkages can be formed efficiently and were found to be sufficiently stable to withstand the further manipulations needed to make the drag-tag useable.

Finding the appropriate reagents and conditions to form five simultaneous peptide bonds in one reaction

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**Table 3. Peptoid Structures, Molecular Mass Confirmation, and Crude Purities**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peptoid Oligomer</th>
<th>Monomer Sequence (Amino-to-Carboxy)</th>
<th>Molar Mass Calc/Found</th>
<th>Purity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30-mer Backbone</td>
<td>FmocGly[NMEG]3(NABG)[NMEG]3,NH2</td>
<td>3818.2/3819.3</td>
<td>77</td>
</tr>
<tr>
<td>2</td>
<td>Tetramer Branch</td>
<td>AcGluOH(NMEG)2,NH2</td>
<td>648.3/648.8</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>Octamer Branch</td>
<td>Ac[NMEG]3GluOH,NH2</td>
<td>1108.6/1109.1</td>
<td>95</td>
</tr>
</tbody>
</table>

*As estimated by analytical reversed-phase HPLC of crude product. All compounds were purified to >99% homogeneity before conjugation reactions.

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**Figure 1.** RP-HPLC chromatogram of the crude products of the grafting reaction between the tetramer branches (2) and the backbone molecule (1). HPLC conditions were C18 250 mm Vydac column, 58 °C, with 10–60% acetonitrile–water (0.1% TFA) over 50 min.
Table 4. Drag-Tag Structures, Molecular Mass Confirmation, and α Values

<table>
<thead>
<tr>
<th>compound</th>
<th>drag-tag</th>
<th>molar mass</th>
<th>α(20)a</th>
<th>α(30)b</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>acetylated</td>
<td>4023.2/4023.5</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>5</td>
<td>tetramer-branched</td>
<td>6964.9/6964.6</td>
<td>12.5</td>
<td>13.7</td>
</tr>
<tr>
<td>6</td>
<td>octamer-branched</td>
<td>9266.1/9271.4</td>
<td>16.4</td>
<td>17.2</td>
</tr>
</tbody>
</table>

a For the 20-base DNA–drag-tag conjugate. b For the 30-base DNA–drag-tag conjugate.

initially proved difficult. Solvents including NMP and DMF were examined. Several peptide bond forming coupling reagents including PyBOP, HATU, and PyBroP were examined (28–30). Optimum results with respect to the efficiency of coupling were obtained utilizing a combination of NMP and PyBroP with DIELA (26). The use of freshly lyophilized chemicals and Aldrich “sure-seal” packaging (where available) and the employment of rigorous Schlenk techniques under a positive nitrogen atmosphere were found to be highly important factors in the achievement of complete grafting coverage of the backbone. The backbone molecule was found to be especially sensitive to air, because it possesses five free amines, which may react with carbon dioxide. After optimization of the reaction conditions, the RP-HPLC chromatograms for the analysis of the crude products of the grafting reactions show a single product peak, later identified by mass spectrometry to be the fully grafted comblike polymer. Under the described reaction conditions, the grafting reaction is highly efficient and goes to completion in only a few hours. Using 6 equiv of branch molecule per amino site on the backbone (30 equiv overall) was found to be sufficient to achieve complete grafting of all five sites. Preparative HPLC fractions were collected and lyophilized to afford a white solid. This protocol does expend a large quantity of the branch reagent, but to ensure that the reaction goes to completion (due to the strict monodispersity needs for ELFSE applications), the expense is deemed acceptable.

Isolated yields for the synthesis of these branched drag-tags are difficult to accurately quantitate since on the scale we performed them (5 mg of backbone), the masses of final product are low (ca. 1 mg). The crude yield as determined by RP-HPLC is ~97% (Figure 1). However, to ensure complete monodispersity, only the product appearing in the central area of the RP-HPLC peak was carried on. The drag-tag conjugate molecules were found to have the correct masses by MALDI-TOF mass spectrometry (Table 4). As described above, the derivatized (branched) molecules were then treated with piperidine and further purified to afford drag-tags with free (and unique) primary amino groups at the N-terminus. These molecules were then ready for sulfo-SMCC activation to enable their conjugation to oligonucleotide primers.

Sulfo-SMCC Activation. The free-amine terminated drag-tags were reacted with the heterobifunctional linker sulfo-SMCC. This reagent consists of two reactive groups: a sulfo-NHS group (reactive toward primary amines) and a maleimide (reactive toward thiols), connected by a cyclohexyl-containing aliphatic linker. The primary amino terminus of the drag-tags reacts with the sulfo-NHS end of the linker. RP-HPLC was then used to remove excess sulfo-SMCC from the reaction mixture, resulting in a high yield (>95%) of drag-tag with a reactive maleimide for subsequent conjugation to thiolated oligonucleotides.

DNA Reduction and Conjugation to Polypeptoids. The 5'-thiolated DNA is obtained as the disulfide dimer, which must be reduced to yield free sulphydryl groups. Complete reduction of the DNA is essential for obtaining a high yield of the desired conjugate molecule. DNA reduction conditions, involving a 40:1 molar ratio of tris(2-carboxyethyl)phosphine (TCEP) to thiolated DNA incubated at 40 °C for at least 90 min, were found to reliably reduce picomole amounts of disulfide-modified DNA with yields in excess of 80%. Dithiothreitol (DTT) may also be used for reduction at slightly alkaline pH, although in our experience a larger excess of DTT must be used with a significantly longer incubation at 40 °C to achieve a comparable level of reduction with these very small amounts of thiolated DNA. Solid-phase reducing agents (resin-bound DTT or TCEP) have given us inconsistent results.

Literature reports as to the reactivity of TCEP toward maleimides are varied (31–33). We have found that the presence of excess TCEP does seem to reduce the efficiency of the conjugation of thiolated DNA to maleimide-activated polypeptoids, and addition of maleimide-activated polypeptoid in slight excess relative to TCEP appears to give optimal conjugation yields (data not shown). Since the polypeptoids in this case are available in large quantities relative to the tiny amounts of DNA required for capillary electrophoresis analysis, the removal of excess TCEP by gel filtration or dialysis was not necessary. Conjugation yields were approximately 70% for polypeptoids conjugated to the 20-base DNA and closer to 95% for polypeptoids conjugated to 30-base DNA.

Free-Solution Capillary Electrophoresis Analysis of DNA–Drag-Tag Conjugates and the Determination of α Values. Capillary electrophoresis with laser-induced fluorescence detection is a powerful and sensitive analytical technique in which charged analytes are separated on the basis of differences in their charge-to-friction ratios or electrophoretic mobilities. Separations are carried out in narrow fused silica capillaries, which allow efficient heat removal and thus enable the use of higher electric fields than can be maintained in conventional slab gel electrophoresis. DNA separations are typically carried out in 50- or 75-μm inner diameter capillaries filled with a viscous polymer solution or sieving matrix. In this study, modifying DNA oligonucleotides with the drag-tags allowed the separation of DNA in free solution with no viscous polymer solution. The elimination of the polymer solution simplifies CE operations and allows the use of narrower capillaries (as small as 25 μm in inner diameter), which afford improved resolution.

The highly negatively charged DNA oligomer component of the drag-tag–DNA conjugates endows each of the bioconjugate molecules with a strong electromotive force during electrophoresis. The polypeptoid portion gives each molecule an additional amount of hydrodynamic drag. The analysis of the drag-tag–DNA conjugates by free-solution electrophoresis allows the determination of the frictional parameter α of the drag-tag, as described in the Introduction (eq 1).

The results of the electrophoretic analyses of the drag-tags conjugated to the 20-base oligonucleotide are shown in Figure 2, and α values calculated from the experiments are shown in Table 4. The acetylated 30-mer 4 gives an α approx 8, whereas the addition of tetramer and octamer branches increases α to about 13 and 17, respectively. Note that the 536-residue protein streptavidin gives an α value that is only twice that of the octamer-branched drag-tag (6), which comprises only 70 monomers. A high degree of monodispersity is a key property for drag-tags, and these drag-tags display excellent purity (>99% by area). Extremely monodisperse drag-tags are necessary
for ELFSE analysis because impurities lead to multiple peaks for each size of DNA. Such extra peaks would confound the results of sequencing or other ELFSE separations demanding high resolution. These results give us good confidence in the viability of making high molar mass peptoids for ELFSE applications.

Interestingly, the relationship between \( \alpha \) and the molecular weight of the drag-tag for these poly(N-methoxyethyl glycine)-based molecules was found to be essentially linear (Figure 3). Table 4 shows the relative molecular weights and averaged \( \alpha \) value for each of these drag-tag conjugates with two DNA primers of different length. This study shows that hydrodynamic drag is not simply a function of the length of the backbone. Rather, drag scales linearly with the total molecular weight or the total number of monomer units. This is in line with previous observations for linear polypeptoids (6) and suggests that the polypeptoids, whether branched or linear, adopt an “expanded” conformation in aqueous solution such that all of the monomer units are hydrodynamically exposed to the solvent during electrophoresis.

**Separation of PCR Products.** The octamer-branched drag-tag (6) was utilized in separating thiolated DNA products from a PCR reaction. PCR was used to generate double-stranded DNA fragments of 50, 75, 100, and 150 base pairs in size. The monodisperse, comblike polymer drag-tag 6 was conjugated to each of the fragments following the PCR reaction, and these conjugate products were successfully separated by CE in free solution, as shown in Figure 4a. The PCR products were denatured prior to analysis and analyzed under denaturing conditions, so the analysis represents single-stranded DNA–drag-tag conjugates. The PCR reaction products were not purified prior to analysis; hence there are some shorter products representing partially amplified PCR products shorter than 50 bases. We left these impurities in the mixture to demonstrate the ability of the drag-tag 6 to resolve small DNA with single-base resolution. The peaks for the 50-base and 75-base fragments are actually split into doublets, indicating the presence of two species (perhaps 50 and 51 and 75 and 76 bases). The reason for this could be due to the propensity of Taq polymerase to generate “stutter” or “shadow” bands, in this case possibly adding an extra base during the PCR reaction (34). Multiple closely spaced peaks are also present for “free” DNA (carrying no drag-tag). There are several different sizes of DNA present in the mixture, including some unreduced disulfide dimers, which may have slightly different mobilities, resulting in the spread of closely

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**Figure 2.** Free-solution capillary electrophoresis analysis of 20-base fluorescently labeled oligonucleotide conjugated to (a) an “unbranched” or acetylated 30-mer drag-tag (4), (b) a tetramer-branched drag-tag (5), and (c) an octamer-branched drag-tag (6). Separations were carried out on a BioRad BioFocus capillary electrophoresis instrument at 40 °C. The running buffer was 50 mM Tris, 50 mM TAPS, 2 mM EDTA, and 7 M urea, pH 8.5, mixed with a 3% (v/v) aqueous dilution of a POP5 solution as a wall coating agent. The fused silica capillary had an inner diameter of 25 \( \mu \)m and a total length of 25 cm (20.6 cm inlet to detector). Samples were introduced by hydrodynamic injection with a pressure–time constant of 5 psi-s. The electric field was 10 kV (400 V/cm) with a typical current of 2.8 \( \mu \)A. The fluorescent label was excited at 488 nm with emission detected at 520 nm.

**Figure 3.** Plot of hydrodynamic drag or “\( \alpha \)” against molecular weight for the 20-base and 30-base DNA–drag-tag conjugates (for all three drag-tags, 4–6).
spaced peaks centered around 6.1 min. For comparison, the separation of these PCR products was performed using a chip-based electrophoresis system (the Agilent 2100 Bioanalyzer) with a polymer solution to provide size-based separation of DNA. The correct sizes of the PCR products were confirmed by comparison to DNA size standards (Figure 4b). The DNA polydispersity around 50 bases correlates well with what was found using the ELFSE technique (compare Figure 4, parts a and b).

The inset of Figure 4 shows a plot of the mobility ratio, \( \mu_0/\mu - 1 \), versus number of bases for each of the major peaks in Figure 4, including the residual primer and impurity peaks. The tallest of the “free” DNA peaks at 6.13 min was chosen as the reference peak for \( \mu_0 \). As can be observed by a simple rearrangement of eq 1, this plot should yield a straight line with slope equal to \( \alpha \). The resulting plot is highly linear (\( R^2 = 0.9997 \)) with a slope of 16.15, in good agreement with the \( \alpha \) values calculated from the separations of the 20-base and 30-base oligonucleotides. The intercept is slightly offset from zero; this may be the result of the fluorescein label, which contains a negative charge and thus slightly affects the electrophoretic mobility of both the conjugates (\( \mu \)) and the free DNA (\( \mu_0 \)).

This separation of PCR products demonstrates the potential usefulness of branched polypeptoid drag-tags for a wide variety of genotyping or “DNA fingerprinting” applications that require size-based separation of DNA. The mixture shown here was intentionally not purified to demonstrate the resolving power of ELFSE for smaller oligonucleotides (the small impurities are easily separated with single-base resolution). This separation methodology could easily be adapted to analyze double-stranded PCR products as well. The highly monodisperse drag-tag and separation method used here offer excellent resolution and peak shape compared to the previous demonstrations of dsDNA separation by ELFSE using streptavidin (8), due to the true monodispersity and favorable chemical properties of these drag-tags. The octamer-branched drag-tag is likely too small for high-resolution separation of much larger PCR products or DNA sequencing fragments, but a similar methodology may be employed to construct much larger branched molecules, for example, using larger branches, a longer backbone, or both.

**CONCLUSIONS**

In this work, we have developed methodology for creating totally monodisperse, comblike, hydrophilic, and water-soluble copolymers, which were successfully synthesized and characterized. The peptide bonds between the branches and the backbone were formed most efficiently using an excess of glutamic acid-terminated branches and PyBroP as a coupling reagent. By conjugating the drag-tags to monodisperse DNA oligonucleotides 20 and 30 bases in length, we then evaluated these branched molecules by free-solution CE as ELFSE drag-tags in terms of their respective frictional parameters (\( \alpha \)). The octamer-branched molecule was also used to successfully separate ssDNA fragments of varying lengths up to 150 bases.

The ELFSE studies of this family of branched drag-tag–DNA bioconjugates reveal a direct linear relationship between molecular weight and hydrodynamic drag. This information will prove useful in the future design of drag-tags for applications in free-solution CE. Achieving significant hydrodynamic drag for poly(NMEG) peptoids may be considered a direct function of molecular weight or the number of monomers, rather than simply the length of the backbone.

We intend to use this structural information about drag-tag design to generate drag-tags with tailored designs that allow the free-solution sequencing of hun-
dreds of bases of DNA. A comblike architecture appears to be an excellent design for drag-tags for the ELFSE technique.

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Supporting Information Available: Additional MALDI mass spectral and ELFSE CE data. This material is available free of charge via the Internet at http://pubs.acs.org.

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