FREE-SOLUTION CAPILLARY ELECTROPHORESIS OF POLYPEPTOID-OLIGONUCLEOTIDE CONJUGATES

Wyatt N. Vreeland and Annelise E. Barron

Department of Chemical Engineering
Northwestern University
Evanston, IL 60208

Introduction

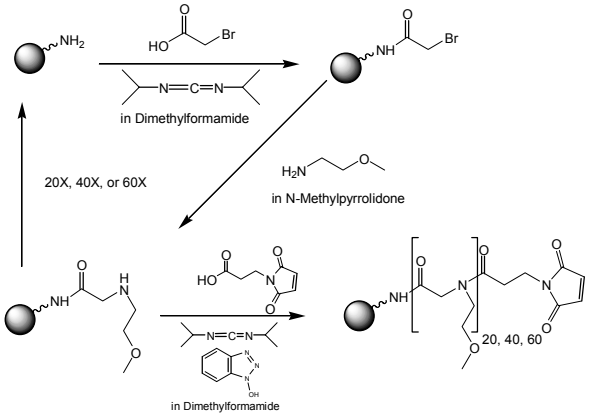
One of the more troublesome aspects of DNA sequencing using capillary electrophoresis is the introduction of the polymeric sieving matrix into the small lumen of the capillary [1]. As instrumentation is further miniaturized, as in electrophoresis chips, loading a replaceable matrix into the electrophoresis chamber becomes difficult to achieve with pressures employable in small geometries. This has led to research into the development of polymeric sieving matrices of minimal viscosity to alleviate this problem [2, 3]. For a long time one solution to this problem has been overlooked; that is to separate DNA in buffer containing no polymeric sieving matrix at all, and approach called End-Labeled Free-Solution Electrophoresis (ELFSE)[4]. DNA does not have a size-dependent electrophoretic mobility in free solution, because both its charge and friction are linear functions of the polymer chain length [5]. Thus, the electrophoretic mobility is constant with respect to length of the DNA molecule. In 1992 Noolandi[6] postulated a method to break this problematic scaling of DNA mobility. He showed that DNA, theoretically, could be electrophoretically separated in free solution by attaching a perturbing entity to one end of the DNA. In theory, this perturbing entity could be any molecule that is monodisperse and has a different charge-to-friction ratio than DNA. Ideally this molecule will be both uncharged and have as high a molecular friction coefficient as possible. We have given this 'perturbing entity' the moniker 'drag-tag' as this aptly describes its function; it serves to add additional hydrodynamic drag to the DNA without adding any additional motive force. To determine the exact chemical properties that impact the efficiency of this electrophoretic separation mechanism we have developed a model synthetic biomimetic polymer system based on poly-*N*-substituted glycines (peptoids) to employ as drag-tags. Here we present the preliminary results of this research.

Materials and Methods

Synthesis of Poly-*N*-substituted glycines (peptoids)

Bromoacetic acid, 1,3-diisopropylcarbodiimide, 2-methoxy-ethylamine, 3-maleimidopropionic acid, piperidine, trifluoroacetic acid (TFA), and dithiotheritol (DTT) were used as purchased from Aldrich chemical company. *N,N'*-dimethylformamide (DMF), acetonitrile and *N*-methylpyrrolidone (NMP) were purchased from Fisher Scientific and used without further purification. Rink amide resin and *N*-hydroxybenzotriazole were purchased from NovaBiochem. Performance Optimized Polymer 6 (POP6) was purchased from Perkin-Elmer Applied Biosystems Division. Oligonucleotides were custom synthesized by Oligos, Etc. Centri-sep coulmns were obtained from Princeton separations. All other reagents were of analytical grade.

A set of poly(*N*-ethoxymethylglycine) homopolymers of 20, 40, and 60 monomers in length with a terminal maleimide functionality were synthesized on a PE-Biosystems 433A® automated peptide synthesizer with a sub-monomer protocol, written in house. Peptoid oligomers were synthesized on 0.1 mmol of Rink amide resin with a substitution of ~0.5 mmol/g. Briefly, after removal of the first Fmoc protecting group from the resin, the following 90-minute monomer addition cycle is performed: The amino-resin is bromoacetylated by adding 4.1 mL of 1.2M bromoacetic acid in DMF plus 1 mL of 1, 3-diisopropylcarbodiimide. The mixture is vortexed in the synthesizer's reaction vessel for 45 min, drained, and washed with DMF (4x7 mL). Next, 6mL of a 1M solution of the 2-methoxymethylamine in NMP is added to the resin and agitated for 45 minutes, drained, and washed with DMF (4x7mL). This process is repeated until the desired number of monomers is added. Then 4.1 mL of a solution of 1.2M maleimidopropionic acid and 1.2M *N*-hydroxybenzotriazole in DMF is added to the resin along with 1mL of 1, 3-diisopropylcarbodiimide, and agitated for 45 minutes. The liquid is then drained and the resin rinsed with dicholoromethane (4x7mL). Next the polypeptoid is cleaved from the resin with 95 vol % trifluoroacetic acid in water (1.5 hours at room temperature). The polypeptoid is then removed from the trifluoroacetic acid by a diethylether/water extraction, and the aqueous phase is frozen at -85°C and lyophilized to yield a yellow-gold oil. This synthetic protocol is outlined below:

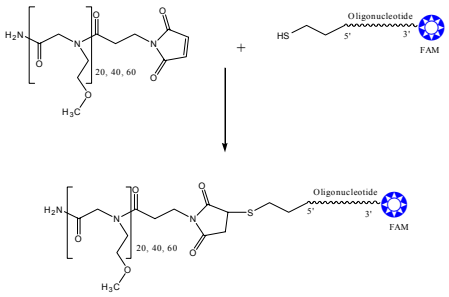


Purification of crude polypeptoids and analysis

Crude peptoids were dissolved in water and analyzed by gradient reversed-phase HPLC on C18 packing (Vydac, 5µm, 300Å, 2.1x250mm). A linear gradient of 10-60% B in A was run over 50min at a flow rate of 0.1mL / min (solvent A=0.1% TFA in water, solvent B=0.1% TFA in acetonitrile) at 60°C; peaks were detected at 220nm. Preparative HPLC was performed on a Vydac C18 column (Vydac, 15µm, 300Å, 22x250mm) using the same solvent and detection systems; peaks were eluted with a linear gradient of 10-60% B in A over 50 minutes at 12 mL / min. The purified peptoids were then analyzed again by the analytical HPLC protocol described above to ensure the molecules were pure. Chromatograms of the crude peptoids are shown in FIGURE 1, and of purified peptoids in FIGURE 2. After purification, fractions were frozen at -85°C and lyophilized.

Conjugation of polypeptoids to oligonucleotides

A mixture of single-stranded oligonucleotides 20 and 21 bases in length, having a 5'-C6 thiol modification and 3'-fluorescein were conjugated to the purified polypeptoids *via* an addition reaction between the 5' thiol modification of the oligonucleotide and the maleimide functionality of polypeptoid to yield a stable thioester linkage. To accomplish this the oligonucleotide was reduced, to eliminate any intermolecular dimer formation as a result of oxidation between the thiols, by first dissolving 12.8 nmol of the oligonucleotide in 30µL of 1X triethylammonium acetate buffer and 4.33 µl of 1M $AgNO_3$ and incubated at 25°C for 30 minutes. Next, 5.78µL of 1M dithiothreitol (DTT) was added and reacted for 5 minutes. The sample was then centrifuged to remove the Ag-DTT precipitate, and the liquid phase was aspirated and placed in a separate tube. The Ag-DTT precipitate was washed twice with 30µl of buffer, centrifuged, and all three liquid phases combined. The liquid containing the DNA was then gel filtered on Centri-Sep columns according to the manufacturer's directions to remove any buffer salts. The eluent from these columns was then immediately frozen in liquid nitrogen and lyophilized. Next each of the purified peptoids was dissolved at a concentration of 12.8µM in 0.1M sodium phosphate, 0.15M NaCl buffer pH=7.2. 10µL of this solution was then added to the lyophilized DNA from the reduction protocol outlined above, and incubated for 20 hours at room temperature. This reaction is outlined below:



Electrophoresis of polypeptoid-oligonucleotide conjugates

The polypeptoid-oligonucleotide conjugates were analyzed *via* capillary electrophoresis using a Bio-Rad CE 3000 equipped with a LIF[2] laser induced-fluorescence detector. Immediately prior to electrophoresis aliquots of each of the samples were combined and diluted 1:100 into deionized formamide, heated to 95°C for 5 minutes, and snap-cooled on ice. Electrophoresis was conducted in fused silica capillaries 40 cm in length (35.4 cm effective length) and 25μm in inner diameter. Before each analysis the capillary was flushed with several column volumes of the running buffer which was 1X TAPS (pH-adjusted to 8.5 with NaOH), 7M urea, and 0.01% (v/v) POP6. Samples were introduced to the column by an electrokinetic injection of 375 V/cm for 20 seconds, followed by electrophoresis at 375 V/cm for 20 minutes. Detection was accomplished *via* the fluorescein label which was excited with the 488nm line of an Ar-Ion laser, with emission detected at 521 nm.

Results and Discussion

The conjugates generated with this synthetic protocol were analyzed by electrophoresis in buffer *without* any polymeric sieving matrix. This analysis leads to separation of each of the six species present in the sample, the results of which are presented in FIGURE 3. To quantify the effective drag imposed on a DNA fragment Slater *et al* [7] developed the following equation:

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

where μ is the electrophoretic mobility of the species of interest, μ_0 is the mobility of unlabeled DNA, N is the number of DNA bases present in the species, β is the charge of the drag-tag in units of charge of one DNA base, and α is the hydrodynamic drag created by the drag-tag in units of drag created by one base of DNA. Since we know from structural information that our drag-tags are uncharged at the pH of analysis we can set β=0, calculate μ and μ_0 from the electropherogram, and determine the value for α for each polypeptoid. We obtain values for alpha that have a linear dependence on chain length (see TABLE 1).

Objects exhibit a hydrodynamic drag during electrophoresis that is a function of both the molecular weight and conformation of the molecule [8]. To make ELFSE a feasible method of DNA separation, drag-tags that produce large values of α are required so that large DNA fragments can be resolved. Therefore it is important that α be a strong function of molecular weight so that a given molecular weight exhibits the maximum drag possible. This dependence is largely determined by the molecule's conformation. The fact that α is a linear function of molecular weight suggests that in fact polypeptoids of sequence have a linear rigid rod-like conformation and that their persistence length is longer than 60 monomers in length.

We believe that polypeptoids are a promising system to determine the important variables that impact the effect of efficiency of ELFSE. This will be important for the development of drag-tags that are large enough to be effectively employed in sequencing separations. The synthesis of polypeptoids is very flexible; virtually any primary amine can be used in the synthesis to yield an incredible variety of side-chains in the polypeptoid[9]. Additionally, this side chains can be incorporated into polypeptoid in a sequence specific manner. We are currently in the process of synthesizing polypeptoids with various side-chain chemical functionalities to determine the impact of side-chain chemistry on important variables such as analyte-wall interactions and hydrodynamic drag. Once the effects of these parameters have been established, the development of larger tags will be pursued to create drag-tags suited to electrophoretic DNA sequencing analysis and capable of separating large DNA fragments.

TABLE 1. α Values for Polypeptoids of Various Lengths

Polypeptoid Chain Length	α value
20	3.8
40	8.4
60	13.2

References

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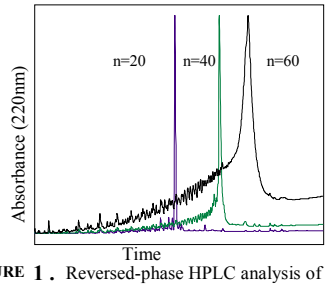

FIGURE 1. Reversed-phase HPLC analysis of polypeptoids.

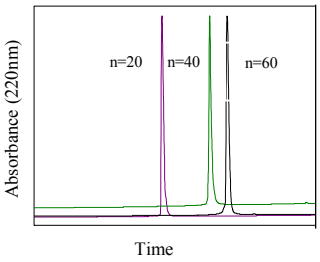

FIGURE 2. Reversed-phase HPLC analysis of purified polypeptoids.

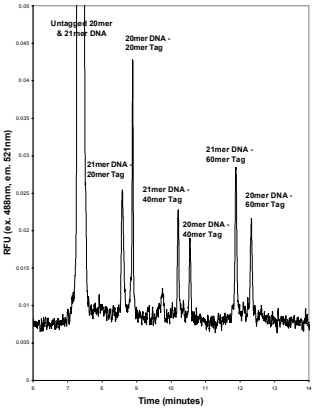

FIGURE 3. Electropherogram of peptoid-oligonucleotide conjugates in free solution.