Profiling Solid-Phase Synthesis Products by Free-Solution Conjugate Capillary Electrophoresis

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Solid-phase synthesis of oligomers, both natural and nonnatural, has proved to be invaluable for the development of many areas of biotechnology. A critical step in the solid-phase synthesis of any oligomer is determining the number and concentration of different constituents present in the product mixture resulting from the synthesis, both before and after purification. Most typically, this analysis is performed by reversed-phase high performance liquid chromatography (RP-HPLC), with the separated components detected by UV absorbance. Recently, we described a novel technique, free-solution conjugate electrophoresis (FSCE), for the high-resolution separation and sensitive laser-induced fluorescence (LIF) detection of uncharged, synthetic polymers, PEG in particular. In this report, we apply this bioconjugate capillary electrophoresis technique to analyze products of the solid-phase synthesis of oligomeric polyamides, namely poly(*N*-substituted glycines), or polypeptoids. When compared to more traditional RP-HPLC analysis, FSCE analysis of oligomeric peptoids results in separation resolutions that are approximately five times higher and separation efficiencies that are increased by 150%. Moreover, when FSCE with LIF detection is applied to the analysis of oligomeric polyamides after HPLC purification, impurities that are not detectable in RP-HPLC analysis are readily separated and detected. With the advent of capillary array electrophoresis (CAE), which allows for automated, parallel analysis of many different samples, we believe that FSCE will be especially applicable to the analysis of combinatorial synthesis products, by allowing researchers to evaluate many different samples in a single, highly parallel, fully automated analysis. This is in contrast to RP-HPLC analysis, in which samples must be analyzed in series.

INTRODUCTION

The solid-phase synthesis of polypeptides (*1*) has, over the past several decades, proven to be an invaluable tool for the study of molecular biology. For example, chemically synthesized polypeptides have allowed biochemists to determine the high-resolution structures of many proteins (*2*−*4*), enabling the elucidation of important biological pathways. In some cases, solid-phase peptide synthesis (SPPS) has allowed the study of proteins or protein fragments that were previously unobtainable in sufficient quantity, if at all, from natural preparations.

More recently, synthetic routes have been developed to various classes of nonnatural, sequence-specific, biomimetic oligomers, including poly-*N*-substituted glycines or "polypeptoids" (*5*−*10*), β-peptides (*11*−*14*), γ-peptides (*15*), oligoureas (*16*), and peptide nucleic acids (*17*). These biomimetic oligomers, based on backbones which are variants of the natural polypeptide (polyamide) structure, with differences also in their side chain chemistries, are of interest for their abilities to adopt stable secondary structures, and for their potential to shed light on factors that endow natural polypeptides with bioactivity (*18*−*21*). It is hoped that nonnatural oligomers can be developed as bioactive, structured peptide mimics, as well as useful materials for other applications in biotechnology such as gene therapy, genetic analysis, and bioseparations.

The ability to synthesize polypeptides on solid-phase support allows the per-monomer coupling efficiencies to be pushed to >99.9%, providing a route to the creation of long oligomers (>40 monomers in length) (*1*). Since the addition of any given monomer in the sequence never goes to 100%, when the final peptide preparation is cleaved from the solid support it represents a mixture of the desired full-length sequence with various deletion products, or if there was incomplete protection of any reactive side chains, even some branched oligomers. A critical step in the production of chemically synthesized polypeptides is the evaluation of the composition of the crude products as they are cleaved from the solid-phase resin. This is typically done by analytical, reversed-phase (RP) HPLC in small-bore columns packed with fine-mesh resin. The level of resolution of the full-length peptide sequence from deletion fragments and side products is generally quite good under the best conditions. HPLC profiling of the crude products of SPPS allows researchers to assess the success of the synthesis, and can be used on intermediary samples to track the coupling efficiency as a function of the length of the growing polypeptide chain. Generally, the predominant peak will be the desired polypeptide if the synthesis has gone well, and can be isolated by preparative RP-HPLC.[1] The final, purified polypeptide preparation will generally be characterized again by analytical RP-HPLC, as well as by

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electrospray ionization mass spectrometry (ESI-MS) to assess its level of purity. For nonnatural oligomers such as polypeptoids and β-peptides, similar approaches to profiling crude products of synthesis and purification are also taken, i.e., RP-HPLC is still the critical and most widely used technique (*6, 22*).

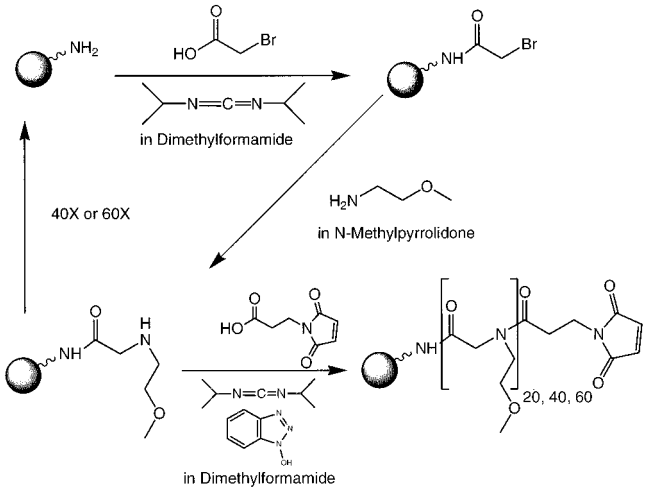
To analyze the overall quality of a crude or purified chemically synthesized polyamide, the different components of the biopolymer sample must be sufficiently separated from one another. These separated components may be quantified according to their relative abundance, their molecular mass, and/or another indicative, quantitative parameter (e.g., UV absorbance at a particular wavelength). When separation is carried out by RP-HPLC, a sample is separated by partitioning between a solid-phase packing contained in the HPLC column and a mobile liquid phase that flows through voids in the packing (*23*). The composition of the liquid mobile phase is often changed throughout the analysis, so that the sample mixture components will each elute in order of increasing hydrophobicity, and are subsequently detected by a UV absorbance detector (*24*).

RP-HPLC results in the oligomer of interest being separated based on the strength of its interaction with the solid phase. The selection of liquid mobile phase and of the chemistry of the column packing determines, in large part, the quality of the separation and of the analysis. For samples that are extremely hydrophilic or hydrophobic, the identification of an appropriate solid phase and method of solvent composition programming to achieve high resolution can be challenging (*25*).

When coupled to a UV absorbance detector, as is typical, the limit of detection (LOD) of the sample in the mobile liquid-phase of an HPLC separation is relatively high when compared to the LOD of highly sensitive laser-induced fluorescence (LIF) detectors that are currently available (*26*). Also, the use of mobile phases with appreciable UV extinction coefficients can severely limit the sensitivity of detection of sample in the eluent of an HPLC column, especially for the UV detection of extremely dilute species in a given sample. Finally, in the analysis of biopolymers that lack a unique chromophore, one must rely on detection of the chromophore of the polymer backbone (214 nm in the case of polyamides). Detecting a chromophore in the polymer backbone, which yields a chain-length dependent molar extinction coefficient, complicates the quantification of sample species, since peak area is then dependent upon both relative abundance and upon chain length.

In the past few years, capillary electrophoresis (CE) has become increasingly popular as a high-resolution, high-sensitivity technique for the separation and analysis of molecules of chemical and biochemical interest including chiral molecules (*27*), proteins (*28*), carbohydrates (*29*), and DNA (*30*−*33*). We recently reported a new capillary electrophoresis technique, which we call free-solution conjugate electrophoresis (FSCE), for the separation and effective mass spectrometry of uncharged poly(ethylene glycol) (PEG) samples (*34*). In that work, molar mass profiling of several different PEG samples was achieved via end-on conjugation of these polydisperse polymers to monodisperse, fluorescently labeled DNA oligomers, followed by free-solution electrophoretic separation of the bioconjugates in a long capillary (*34*). We show here that this technique can be extended to the separation and analysis of both crude and purified solid-phase synthesis products.

Scheme 1



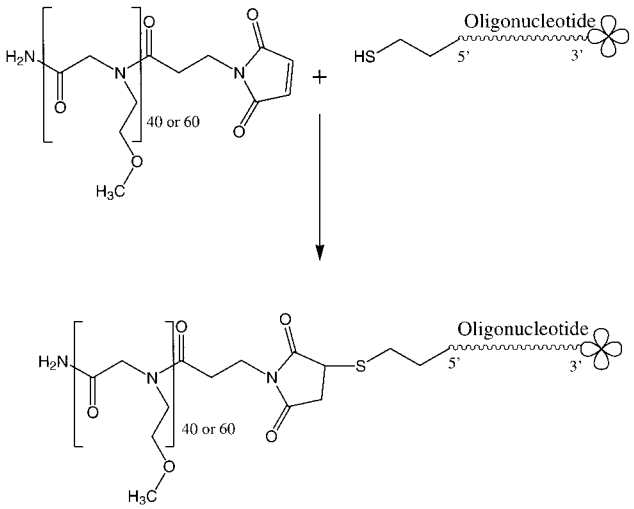
In particular, we describe the application of FSCE to the analysis of both unpurified and purified oligomers produced via chemical synthesis. This method, we believe, provides an interesting alternative to HPLC for the characterization of chemically produced polyamides. The novel separation mode that we demonstrate is accomplished by end-on conjugation of each of the species present in a complex oligomer mixture with a monodisperse, fluorescently labeled DNA oligomer. The resulting DNA−oligomer conjugates are then separated by free-solution capillary electrophoresis. Specifically, we apply FSCE to a class of biomimetic molecules that are remarkably similar to polypeptides, called poly-*N*-substituted glycines, or *polypeptoids*. Exploiting the extreme sensitivity of LIF detection and the powerful resolving power of capillary electrophoresis, FSCE allows for accurate and sensitive separation of various species present in a complex sample mixture resulting from a solid-phase synthesis.

EXPERIMENTAL SECTION

Polypeptoid Synthesis. The synthesis of the oligomer samples of interest, in particular the poly-*N*-substituted glycines or "polypeptoids" used in this study, has been described elsewhere (*5*). The synthetic protocol for the particular molecules studies here is depicted in Scheme 1. All reactions were carried out on an ABI 433A automated peptide synthesizer (Applied Biosystems, Foster City, CA) with in-house modifications to the control software. Briefly, 0.25 mmol of Fmoc-rink amide resin (Nova Biochem, San Diego, CA) were deprotected by treatment with 20% (v/v) piperidine (Applied Biosystems, Foster City, CA) in dimethyformamide (DMF) (Fisher Scientific, Itasca, IL), in two consecutive 15-min treatments. The oligomer chain was then assembled with alternating cycles of bromoacetylation step and amine displacement of the bromine from the *N*-terminal alkyl halide. The bromoacetylation was accomplished by mixing 4.3 mL of 1.2 M bromoacetic acid (Aldrich, Milwaukee, WI) in DMF and 1 mL of diisopropylcarbodiimide

[1] Abbreviations: CAE, capillary array electrophoresis; CE, capillary electrophoresis; ESI-MS, electrospray ionization mass spectrometry; epoxy-pDMA, poly(dimethylacrylamide-*co*-allyl glycidyl ether); FSCE, free-solution conjugate electrophoresis; LIF, laser-induced fluorescence; LOD, limit of detection; MALDI-TOF, matrix-assisted laser desorption ionization−time-of-flight; PEG, poly(ethylene glycol); RP-HPLC, reversed-phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis.

Scheme 2



(Aldrich, Milwaukee, WI) with the resin. The mixture was then vortexed for 45 min, the liquid drained, and the resin rinsed 4 times with 7 mL of DMF. Next, the resin was mixed with 4 mL of 1 M methoxyethylamine (Aldrich, Milwaukee, WI) in *N*-methylpyrrolidone and vortexed for 45 min to introduce the methoxyethyl side-chain moiety. The liquid was then drained, and the resin was again rinsed four times with 7 mL of DMF. These two reaction cycles were then alternated until the polypeptoid was of the desired length.

A reactive *N*-terminal maleimide functionality was attached to the amino terminus of the polypeptoid while it was still on the resin, to allow for facile conjugation to DNA oligomers, as depicted in Scheme 2. This was accomplished by mixing the resin with 500 μL of 1.2 M maleimidopropionic acid (Fluka, Buchs Switzerland) in DMF and 144 μL of diisopropylcarbodiimde, vortexing the resin slurry for 45 min, and rinsing the resin four times with 3 mL of DMF. Finally, the polypeptoid was cleaved from the solid support by treatment with 95% (v/v) trifluoroacetic acid (Aldrich, Milwaukee, WI) in water for 30 min. The polypeptoid-containing liquid was then passed through a filter to separate it from the solid support. Next, the crude polypeptoid solution was extracted with a water−diethyl ether mixture, and the aqueous layer retained, frozen, and lyophilized to yield a golden, viscous oil.

RP-HPLC Analysis Conditions. Unpurified and purified peptoids were dissolved in water and analyzed by RP-HPLC on C18 packing (Vydac, 5 μm, 300 Å, 2.1 × 250 mm). A linear gradient of 10−60% B in A was run over 50 min at a flow rate of 0.1 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) at 60 °C; peaks were detected by UV absorbance at 214 nm. Preparative HPLC was performed on a Vydac C18 column (Vydac, 15 μm, 300 Å, 22 × 250 mm) using the same solvent and detection systems; peaks were eluted with a linear gradient of 10−60% B in A over 50 min at 12 mL/min.

Polypeptoid−DNA Conjugation Conditions. Prior to conjugation with a monodisperse, fluorescently labeled DNA oligomer, each polypeptoid was purified from non-peptoid impurities remaining from the cleavage reaction by preparative HPLC. This was necessary because the particular solubility properties of this peptoid sequence prevented us from using ether precipitation to remove small-molecule contaminants of the cleavage products. We intend to develop a one-step, on-resin conjugation with the DNA label that will allow this step to be bypassed in the future. To accomplish the necessary purification for this study, several milligrams of unpurified polypeptoid were loaded onto the preparative HPLC column; after the elution of the initial void volume peak, the entire eluent of the HPLC analysis was collected for FSCE analysis of unpurified polypeptoids. For the FSCE analysis of HPLC-purified polypeptoids, only the desired peak, representing the full-length product, was collected. Subsequently, each sample was frozen and lyophilized from the HPLC buffer to yield a light gold, viscous oil.

Poly(*N*-methoxyethyl)glycines 40 and 60 monomers in length were each conjugated end-on to poly(thymidine)-deoxyribonucleic acid 20 bases in length (Oligos, Etc., Wilsonville, OR). DNA samples had been prepurified using gel electrophoresis by the vendor, to ensure that each DNA oligomer was monodisperse. Moreover, each DNA oligomer included a fluorescein dye on its 3′ terminus to allow for LIF detection, and a thiol (SH) functionality on its 5′ terminus to allow for conjugation with the maleimide functionality of the amino terminus of the polypeptoid. This conjugation forms a stable thioether linkage between the DNA and the polypeptoid (Scheme 2).

To ensure that the 5′-thiol terminus of the DNA was optimally reactive with the maleimide terminus of the polypeptoid, the DNA was first chemically reduced, as recommended by the vendor and previously described (*34*).

The conjugation reaction was accomplished by dissolving a given polypeptoid sample to a concentration of 12.8 mM in 0.1 M sodium phosphate buffer, 0.15 M NaCl, pH = 7.2, and adding 10 μL of each of the 12.8 mM polypeptoid solutions to the lyophilized DNA powder from the above reduction protocol. This solution was incubated at room temperature for 24 h. Finally, the resultant mixture was diluted 1:300 in distilled, deionized H_2O immediately prior to capillary injection and analysis.

Capillary Electrophoresis Analysis Conditions. All capillary electrophoretic separations were conducted in 25-μm inner-diameter fused silica capillaries, 100 cm in total length (95.4 cm effective length) (Polymicro Technologies, Phoenix AZ), and filled with one of two buffers. For the analysis of unpurified polypeptoids, 1X TTE + 7 M urea (50 mM *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid, 50 mM Tris, 2 mM ethylenediaminetetraacetic acid, 7 M urea), pH = 8.5 (Amresco, Solon, OH) buffer was used, whereas for the analysis of purified polypeptoids, 1X TTE buffer was used. All buffers were prepared with 18 MΩ distilled, deionized water (E-pure, Barnstead, Dubuque, IA). The internal surface of the capillary was dynamically coated with an adsorbed layer of a poly(dimethylacrylamide-*co*-allyl glycidyl ether) (epoxy-pDMA) to reduce electroosmotic flow mobility to negligible levels (*35*). All analyses were performed using a BioFocus 3000 Capillary Electrophoresis system equipped with a laser-induced fluorescence detector (BioRad, Hercules, CA). At the beginning of each day of analysis, the capillary was flushed for 15 min with 1 M NaOH, followed by a 5-min water rinse, and then flushed for 20 min with a 0.1% (w/v) solution of epoxy-pDMA in water. All capillary rinses were done with a syringe pump at a flow rate of 100 μL/h. Electroosmotic flow after capillary coating by this method was measured to be 3.0×10^{-10} m^2/(V·s) by the method of Williams and Vigh (*36*).

Prior to each analysis, the capillary was flushed with several column volumes of the electrophoresis buffer. Immediately prior to sample injection, the injection end

of the capillary was briefly dipped into deionized water to remove residual buffer salts from the outer surface of the capillary. Sample was introduced into the capillary by an electrokinetic injection at 150 V/cm for 2 s. Electrophoresis was conducted at 300 V/cm until all peaks had eluted. Detection of the analytes was achieved with excitation of the 3′-fluorescein DNA label using the 488 nm line of an argon-ion laser, and emission detected at 521 nm.

Data Analysis. All peak parameters and migration times were determined with the PeakFit software package (SPPS, Inc., Chicago, IL).

RESULTS AND DISCUSSION

The separation mechanism of FSCE derives from the different bioconjugate properties created by the covalent linkage of the polyamide molecules to monodisperse DNA oligomers. The highly charged DNA oligomer component of the conjugate endows each molecule with an equal electromotive force during electrophoresis, while the less charged polyamide component gives each molecule a unique amount of hydrodynamic drag. Hence, the electrophoretic mobility of each bioconjugate is a precise function of the length and nature of the polyamide "tail", allowing for free-solution electrophoretic separation of each of the conjugates. The advantages of this approach can be illustrated by carrying out a direct comparison of the results of RP-HPLC and FSCE analysis of a set of model peptoid−DNA conjugates. For this study, we selected a very simple model polypeptoid molecule for illustrative purposes, composed of N-substituted glycine monomers with an uncharged, hydrophilic methoxyethyl side-chain moiety.

Electropherograms and Chromatograms for the Analysis of Unpurified Polypeptoids. Figure 1a and 1b show the results of RP-HPLC analyses of 40-mer unpurified peptoid and 60-mer unpurified peptoid oligomers prepared by solid-phase synthesis, respectively. In RP-HPLC analysis, the constituents of the complex samples are separated on the basis of their effective hydrophobicity. For peptoids composed of this particular monomer (*N*-methoxyethyl glycine), the effective hydrophobicity of the molecule increases as the peptoid chain increases in length. Thus RP-HPLC, in this case, separates the different constituents of the complex sample in order of increasing chain length. The major peak in each figure, while not fully resolved from other sample components, represents the full-length sequence (as confirmed by electrospray mass spectrometry of the material in this peak).

Figures 2a and 2b present the results of the FSCE analyses of a 20-base DNA + 40-mer unpurified peptoid conjugate, and of a 20-base DNA + 60-mer unpurified peptoid conjugate, respectively. Although HPLC and FSCE separate the constituents of the complex sample via different mechanisms, the qualitative results of the two separation mechanisms are similar. The FSCE analyses, however, result in the constituents of the complex sample mixture being resolved more fully when compared to RP-HPLC analyses of the same molecules. This is a result of the high selectivity of capillary electrophoresis and the exquisite sensitivity of LIF detection. The initial group of peaks in Figure 2a eluting at approximately 24 min represents various "free" residual DNA species from the conjugation reaction that were not conjugated to any peptoid oligomers. This peak is not present in Figure 2b, as the concentrations were adjusted

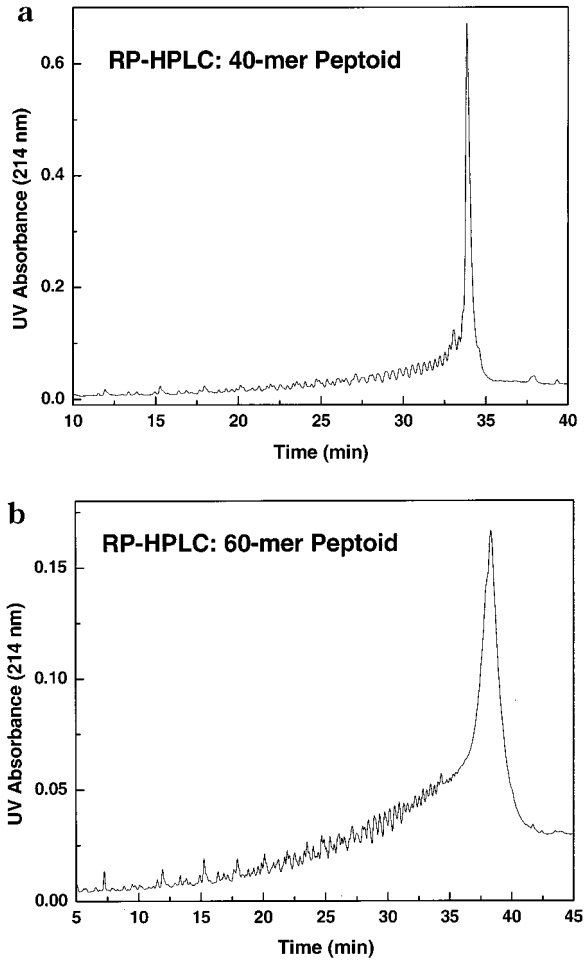


Figure 1. (a) RP-HPLC separation of a crude (unpurified) 40-monomer poly(*N*-methoxyethyl glycine). Conditions: C18 packing (Vydac, 5 μm, 300 Å, 2.1 × 250 mm). A linear gradient of 10−60% B in A was run over 50 min at a flow rate of 0.1 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) at 60 °C; peaks were detected at 220 nm. (b) RP-HPLC separation of an unpurified 60-monomer poly(*N*-methoxyethyl glycine). Conditions as in Figure 1a.

in this reaction so that there was virtually no residual, unreacted DNA. In both Figures 2a and 2b, numerous small peaks representing DNA−peptoid conjugates, eluting in order of increasing peptoid chain length, follow the initial "free DNA" peak. The latest eluting, major peak represents the conjugate with the largest peptoid, which is the full-length desired product (confirmed by mass spectrometry).

The presence of a single fluorophore on the 3′ terminus of each of the DNA−peptoid conjugates allows one to calculate the molar percentage of each component in the original peptoid sample, simply as the ratio of the area of the peak representing that particular peptoid component to the area of all DNA−peptoid conjugate peaks in the electropherogram. Thus, the percent yield of the full-length product is simply the ratio of the area under the last eluting major peak to the sum of the areas under all DNA−peptoid conjugate peaks. Electrokinetic injections, which were used in this study, can cause a bias in the amount of higher-mobility sample components introduced into the capillary. Higher-mobility species (i.e., conjugates with short peptoid components) will tend to be injected to a greater extent than lower-mobility fragments. Formally, one must correct for this bias to obtain accurate quantitation of the relative abundance of each species

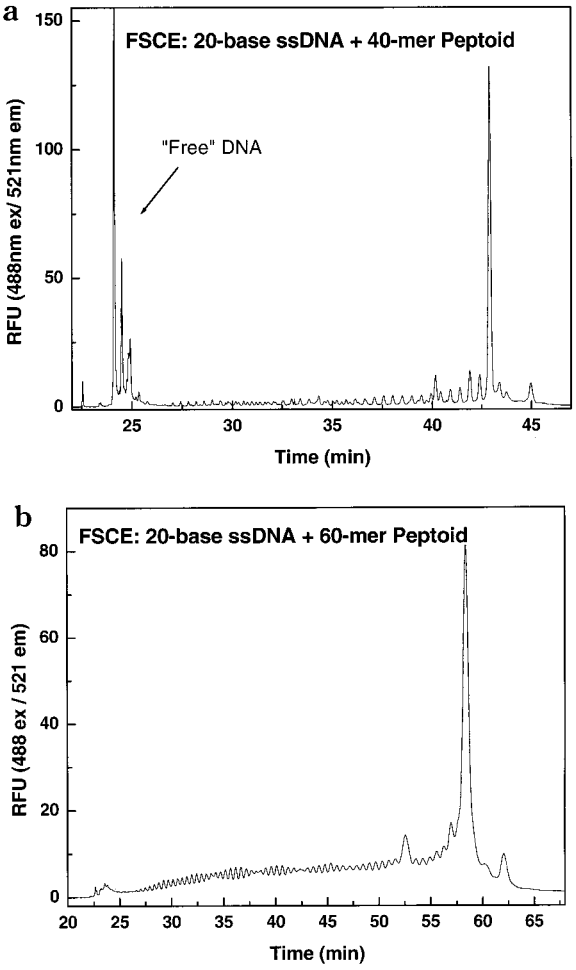


Figure 2. (a) FSCE separation of an unpurified 40-monomer poly(*N*-methoxyethyl glycine) using a 20-base ssDNA "engine". Conditions: buffer 1X TTE + 7 M urea, pH 8.5. Field Strength 300 V/cm, current ~ 4 μA. Capillary 100 cm × 25 μm (95.4 cm effective length). LIF detection, 488 nm excitation, 521 nm emission. Injection: 150 V/cm, 2 s. (b) FSCE separation of an unpurified 60-monomer poly(*N*-methoxyethyl glycine) using a 20-base ssDNA "engine". Conditions are as in Figure 2a.

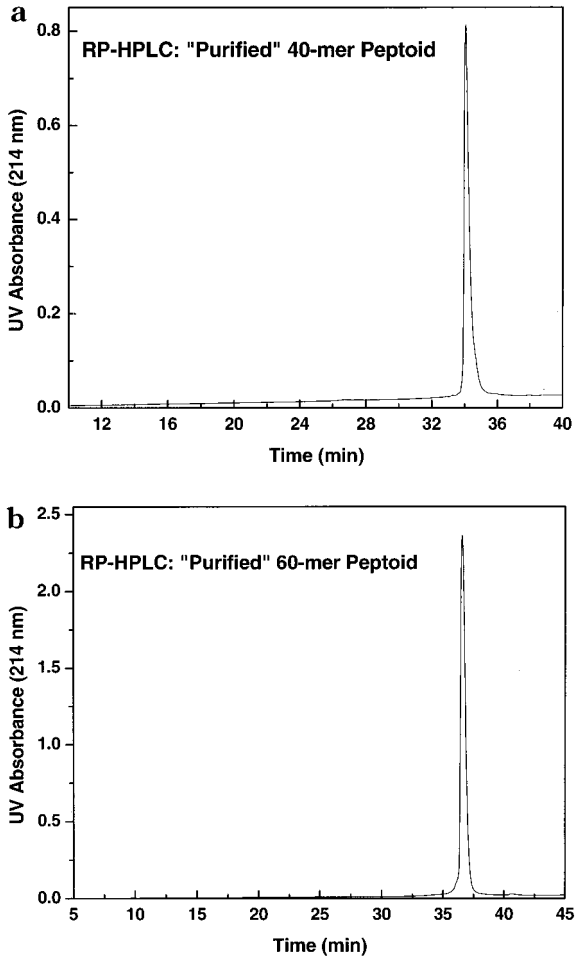


Figure 3. (a) RP-HPLC analysis of a RP-HPLC-purified 40-monomer poly(*N*- methoxyethyl glycine). Conditions as in Figure 1a. (b) RP-HPLC analysis of a RP-HPLC-purified 60-monomer poly(*N*-methoxyethyl glycine). Conditions as in Figure 1a.

present in the sample. However, in previous FSCE studies, we have determined this bias to be negligible (*34*).

Chromatograms and Electropherograms for the Analysis of Purified Polypeptoids. Figures 3a and 3b show the analytical HPLC chromatograms obtained for 40- and 60-mer polypeptoids *after* preparative HPLC purification. Notice that both RP-HPLC analyses show a single, large, relatively Gaussian peak, leading to the conclusion that the polypeptoid samples are essentially pure after the preparative HPLC purification step. That is, virtually no impurities can be detected by RP-HPLC with UV absorbance detection.

These HPLC results are in contrast to Figures 4a and 4b, which show the results of the FSCE analysis of the same, purified 40- and 60-mer peptoid samples after conjugation to 20-base DNA oligomers. Comparison of Figures 3a and 3b with Figures 4a and 4b shows the superior separation performance and sensitivity of FSCE with LIF detection for the detection of sample impurities, when compared to standard RP-HPLC with UV detection. Impurities that are present in purified polypeptoid samples are not observed in HPLC analysis, yet are readily detected by FSCE analysis and represent substantial contamination of the desired peptoid (41% and 47% in Figures 4a and 4b, respectively). Thus, FSCE can lead to a more accurate and sensitive characterization of the absolute purity of a polyamide sample than is possible with standard RP-HPLC. This may be important for the evaluation of polypeptide or polypeptoid purity for applications, such as peptidomimetic pharmaceuticals, where the ultimate purity of an oligomer sample can impact not only its performance, but also the side effects it engenders.

Plate Heights. The number of theoretical plates achievable in a given separation length provides a quantitative measure of the efficiency of the separation. For separation techniques such as HPLC and CE, which are configured in a "finish-line" detection configuration in which the analytes migrate through a stationary detector, separation efficiency is manifested as the peak width. A low number of theoretical plates indicates that the separation technique or hardware has effects that are limiting the separation of the individual components in the complex sample mixture. A plot of the number of theoretical plates as a function of peptoid size, for both the HPLC and FSCE separations, is presented in Figure 5, for the 40-mer polypeptoid. In both FSCE and HPLC analyses, the last major eluting peak was assumed to be the full-length synthesis product and is assigned a peak number corresponding to the number of monomers in the polypeptoid (e.g., 40 for the 40-mer polypeptoid, 60 for the 60-mer polypeptoid). Peaks were then counted backward (i.e., decreasing in time) and assigned a peak number of one less than the peak that eluted immediately after it. Plate efficiencies were determined using the

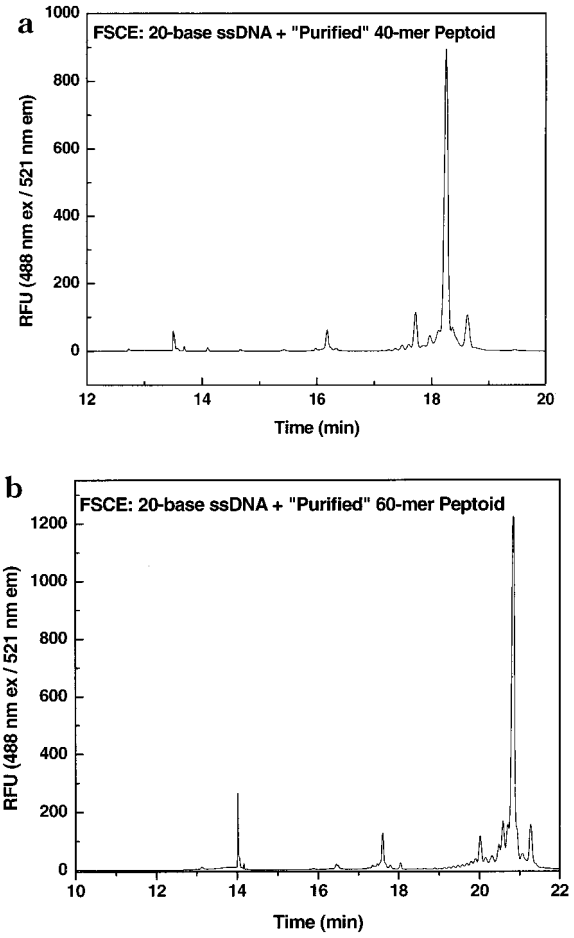


Figure 4. (a) FSCE analysis of the sample seen in Figure 3a using a 20-base ssDNA "engine". Conditions: Buffer 1X TTE, pH 8.5, other conditions as in Figure 2a. (b) FSCE analysis of the sample seen in Figure 3b using a 20-base ssDNA "engine". Conditions as in Figure 4a.

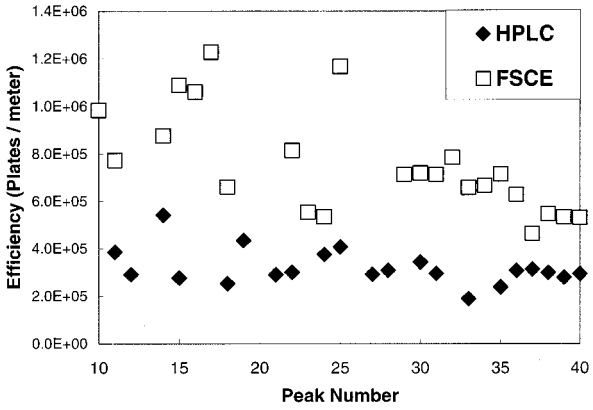


Figure 5. Plot of separation efficiency as a function of peak number for the FSCE separation (open squares) and RP-HPLC separation (closed diamonds) of the 40 monomer poly(*N*-methoxyethyl glycine) seen in Figures 1a and 2a. Peak data obtained with PeakFit software.

method described by Ren et al. (*37*). As can be seen in the figure, the average plate efficiency of the FSCE analysis is 2.5 times greater than that of an RP-HPLC, with several peaks displaying efficiencies well over 1×10^6 plate per meter. Higher average plate efficiency indicates that FSCE analysis produces peaks that are narrower than in HPLC; hence, we can conclude that fewer dispersive effects are limiting the FSCE separation when compared to more traditional RP-HPLC chromatographic separation techniques. Further, this shows that a greater number of species can be resolved in a given time window due to their narrower representative peaks.

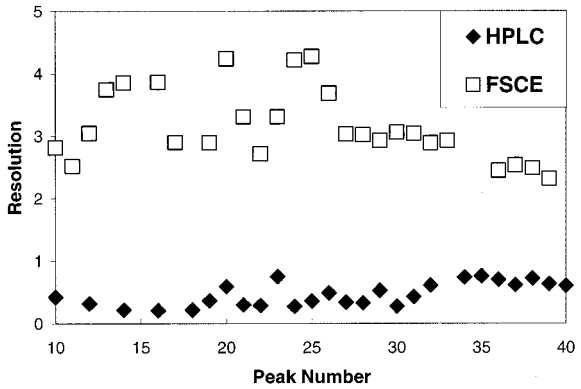


Figure 6. Plot of separation resolution as a function of peak number for the FSCE separation (open squares) and RP-HPLC separation (closed diamonds) of the 40 monomer poly(*N*-methoxyethyl glycine) seen in Figures 1a and 2a. Peak data obtained using PeakFit software.

Resolution. Resolution provides an overall, quantitative measure of how well each of the components in the complex sample is separated. Resolution of neighboring peaks can be calculated as:

$$R = \frac{1.18(t_b - t_a)}{(\mathrm{FWHM}_a + \mathrm{FWHM}_b)} \quad (1)$$

where t_a and t_b are the elution times of peaks two adjacent peaks a and b, respectively, and FWHW_a and FWHW_b are the full temporal width at half the peak height of peaks a and b, respectively (*37*). Figure 6 presents the resolution of both HPLC and FSCE separations methods as a function of peptoid size for the 40mer polypeptoid. Peaks were assigned using the methodology explained in the Plate Heights section. As can be seen, the average resolution of the FSCE analysis is approximately 5 times that of RP-HPLC analysis and allows components of the complex sample mixture to be more fully resolved. Increased resolution means that the peaks are further separated from one and other and therefore are less likely to overlap in a given analysis. Peak separation leads to more accurate quantitation of each species present and thereby a more precise evaluation of the composition of the sample mixture.

The application of free-solution conjugate electrophoresis (FSCE) to the analysis of chemically synthesized oligomers, as we have demonstrated it here with maleimide−thiol linking of the DNA to the analyte, should be directly applicable to virtually any chemically synthesized oligomer, in particular polypeptides. As long as those peptides do not contain reactive thiols (cysteines), and the cleavage conditions to remove the polyamide from the solid support do not require the use of chemical that will react with the terminal maleimide, FSCE should be applicable. The addition of the maleimide functionality to the terminus of the oligomer is accomplished with standard chemicals for the synthesis of polyamides, such as peptides and peptoids and is therefore compatible with existing synthesis chemistries and protocols. However, the peptoid sequence chosen for this study did not require the use of side-chain protecting strategies, simplifying the compatibility of the maleimide linking protocol with the cleavage conditions. If more complicated and diverse side-chain chemistries are present in the polyamide analyte, alternative linking strategies will have to be

implemented to actualize FSCE profiling. Additionally, the solubility properties of this particular peptoid sequence prevented the use of standard ether precipitation workup techniques; thus, FSCE analysis was preceded by an RP-HPLC semipurification to remove small molecule contaminants. However, with optimization of chemical linking strategies to be insensitive to these contaminants, this procedure could be obviated.

CONCLUSIONS

FSCE analysis, when applied to chemically produced oligomers, results in high-resolution and high-efficiency separations. The average separation efficiency is 2.5 times greater and resolutions are 5 times higher than those obtained with RP-HPLC. When applied to HPLC-purified oligomer samples, FSCE allows for the separation and detection of impurities that are not observed in the HPLC analysis of the same samples. Thus, FSCE offers an attractive alternative for the analysis of chemically produced oligomers that may be challenging to analyze via more traditional chromatographic methods. With further development and optimization it seems likely that FSCE, with its orthogonal separation mechanism, could serve as a useful complement to HPLC that will allow for extremely sensitive and accurate characterization of polyamide samples including polypeptides of therapeutic interest. Moreover, with the advent of capillary array electrophoresis (CAE) instruments, now available commercially, FSCE with a modified conjugation strategy could allow the products of combinatorial solid-phase peptide synthesis to be analyzed in parallel. This would be impossible by HPLC, where typically only a single column is available and samples must be analyzed in series.

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