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Nitrogen-doped carbon dots aid in the separation of ssDNA molecules of different length by capillary transient isotachophoresis (ctITP) with laser-induced fluorescence (LIF) detection

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ABSTRACT

This study demonstrates a novel application of nitrogen-doped carbon dots (NCDs) to enable the separation of different lengths of single-stranded DNA (ssDNA) by eletrokinetic means. Carbon dots have recently found widespread application in the fields of sensing, diagnostics, and healthcare due to their biocompatibility and low toxicity. In light of growing interest in the use of ssDNA aptamers over antibodies in the fields of biosensor development and drug delivery, it is important to establish a simple and effective method for aptamer separation. In this study, we employed NCDs as buffer additives in a capillary electrophoresis (CE)-based method, giving rise to the separation of FAM-labeled ssDNA samples ranging from 32 to 100 bases in length, with resolutions ranging from 1.30 – 1.77. In particular, we adopted a capillary transient isotachophoresis (ctITP) system with laser-induced fluorescence (LIF) detection, with both the separation and sample buffers modified by the addition of 30 μg/mL NCDs. These nanomaterials were prepared by a simple hydrothermal method from a mixture of citric acid and ethylenediamine. The NCDs themselves are highly fluorescent and photostable. As components in the background electrolyte, they did not interfere with the fluorescence emission of the FAM-labeled DNA samples. Under the conditions employed, no separation could be achieved in the absence of the NCDs nor with undoped CDs. The results show that NCDs function as buffer additives capable of enhancing electrokinetic-based separations of ssDNA, and hence, provide a new application for these carbon nanomaterials.

1. Introduction

The separation of ssDNA is of great importance in the fields of biochemistry, chemical biology, and molecular biology [1]. Traditionally, slab gel electrophoresis is the method of choice for the separation of different sizes of DNA. However, this method has several limitations such as low sensitivity, low resolution, long analysis times, and the phenomenon of gel deformation under high applied voltage [2]. Capillary electrophoresis (CE) offers many advantages over its slab gel counterpart. such as small sample volume, fast analysis times, high resolution, and high sensitivity when paired with laser-induced fluorescence (LIF) detection. In particular, this work uses capillary transient isotachophoresis (ctITP) as the CE-based separation mode, in which a discontinuous buffer system is employed. This methodology can provide improved resolution due to the focusing effect produced at the interface between the sample and separation buffers [3-5]. Samples are preconcentrated on-column under the influence of a leading and trailing electrolyte in the ctITP system, which allows the amount of sample to be maximized without hurting the resolution [6].

Free solution CE-based methods (including ctITP) rely on differences in the charge-to-size ratio of analytes to effect their separation. Unfortunately, due to similar charge-to-size ratios possessed by DNA molecules in the 10-150 base range, free solution CE-based methods are not able to afford the needed separation, and so CE with gels and coated capillaries is typically employed [7]. Without such modifications, DNA samples in this size range are expected to co-elute under free-solution conditions. Unique to this work is the addition of nitrogen-doped carbon dots (NCDs) to both the separation and sample buffer in a free solution ctITP system. By incorporating these nanomaterials uniformly throughout the system, the ctITP focusing effect is not disrupted, but rather, it is further enhanced due to differential interactions of the NCDs with different sizes of ssDNA molecules in the sample. As such, differential mobilities are observed for DNA samples in the range of 32-100 bases even under free solution conditions.

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Carbon dots (CDs) are quasi-spherical fluorescent nanoparticles with a size typically smaller than 10 nm. The photoluminescence properties of these materials are attributed to the preponderance of sp^3 hybridized carbon atoms resulting from the CD synthesis process. Carbon dots offer several advantages over inorganic nanomaterials, including stability, ease of functionalization, low toxicity, biocompatibility, low cost, ease of synthesis, and more. They are highly soluble in water as they contain carboxylate functional groups on their surface [8,9]. They can interact with a variety of analytes by way of π-π stacking, hydrophobic interactions, hydrogen bonding, and/or electrostatic interactions [10]. Furthermore, the properties of CDs can be altered by the addition of dopants during synthesis, or by surface modification following synthesis. Nitrogen doping is popular, and has been shown to result in higher fluorescence quantum yields [11]. For these many reasons, CDs are particularly attractive as fluorescent nanomaterials in the area of biosensing, where they have been used to aid the diagnosis of disease, especially cancer [12-13]. However, CDs have attracted less attention as separation tools, and so their use as a buffer modifier in CE-based DNA separations – as presented herein – represents a novel application of these important carbon nanomaterials. It is noted that in recent studies, graphene oxide (GO) has been used to make DNA-based optical sensors, whereby GO is shown to interact with ssDNA by π- π interactions [14]. Also, differences in the binding affinity of GO with various lengths of ssDNA has been demonstrated [15]. In this prior work, short ssDNA was shown to interact more strongly with GO than long ssDNA. However, these studies did not demonstrate the free-solution separation of ssDNA samples based on their corresponding sizes.

Aptamers are single-stranded DNA or RNA oligonucleotides (10-100 bases) that can bind with high specificity to target molecules. Recent studies have shown that aptamers can be a suitable alternative to antibodies as an analytical tool [16]. Moreover, they can function as drug delivery agents and therapeutic tools [17]. Aptamer sequences have been identified for targets of various sizes and types, including proteins, microbes, whole cells, and small molecules [18].

Although CE-based methods have been reported for aptamer separations, the majority of such studies have focused on the resolution of free DNA from target-bound aptamers [19-20]. Still other CE-based methods have used polymer or cellulose derivatives as buffer additives for aptamer separations based on their sizes [21-22]. However, the separation of different lengths of ssDNA samples has not been achieved by free-solution CE methods.

In this work, we have introduced nitrogen-doped carbon dots (NCDs) as buffer additives to effect the separation of ssDNA samples ranging in size from 32-100 bases in a free-solution, eletrokinetic-based method. To the best of our knowledge, using carbon dots as buffer additives for the separation of ssDNA samples based on size (number of bases) has not been previously reported. Using carbon nanomaterials, which differentially interact with oligonucleotides and thus result in their differential migration under the influence of an applied electric field in a discontinuous buffer system (as provided by the ctITP conditions employed herein), represents a wholly new application for these materials.

2. Materials and methods

2.1. Chemicals and reagents

Citric acid (> 99.0%), ethylenediamine (> 99%), and glycine (BioUltra grade, ≥ 99.0% NT) were purchased from Sigma Aldrich (St. Louis, MO, USA). All DNA samples were custom synthesized with a 6-fluorescein amidite (FAM) fluorescent label at the 5′ end and purchased from Integrated DNA Technologies, Inc. (IDT) (Coralville, IA, USA), including: 32-base ssDNA (5′-/56-FAM/AG ACA AGG AAA ATC CTT CAA TGA AGT GGG TCG-3′); 61-base ssDNA (5′- /56-FAM/AGC ACA AGC AGT ACT AAG TCC GTG GTA GGG CAG GTT GGG GTG ACT GAC AGC GTA TGG TGA G -3′); and 100-base ssDNA (5′- /56-FAM/CC TCT CTA TGG GCA GTC GGT GAT AGA CAA GGA AAA TCC TTC AAT GAA GTG GGT CGT AAG GAG TTC AAT ATC TGA GTC GGA GAC ACG CAG GGA TGA GAT GG - 3′). Tris(hydroxymethyl)aminomethane (“tris,” Proteomics grade) was purchased from VWR Life Science (AMRESCO, Solon, OH, USA). Ultrapure water obtained from a Milli-Q water system (Millipore Corporation, Billerica, MA, USA) was used for buffer preparation.

2.2. Buffer and sample preparation

Distinct separation and sample buffers are essential for ctITP, in order to effect the stacking and sample concentration characteristic of the method. These studies employed a separation buffer (referred to as Tris-Gly) containing 15 mM tris and 500 mM glycine (pH 7.92), and a sample buffer (referred to as Tris-HCl) containing 50 mM tris, adjusted to pH 8.0 by the addition of 1.0 M HCl. The Cl^- anion in the Tris-HCl sample buffer functioned as the leading electrolyte, while the glycine in the Tris-Gly separation buffer functioned as the trailing electrolyte. Buffers were filtered through a 2.0 µm nylon syringe filter prior to use. The appropriate volume of an aqueous solution of carbon dots (prepared as described in Section 2.3) was added equivalently to sample and separation buffers, to achieve a final concentration of NCDs of 30 µg/mL.

Stock solutions of ssDNA were prepared by adding the appropriate volume of sample buffer to the synthesized ssDNA sample to yield a concentration of 2 µM DNA. The stock solutions of ssDNA were heated at 95°C in a hot water bath for 5 min and allowed to cool back down to room temperature, to facilitate proper folding. DNA stock solutions thus prepared were stored in the refrigerator until needed. Just prior to each experiment, ssDNA samples were prepared by adding the appropriate volume of DNA stock solution to sample buffer.

2.3. Preparation and characterization of nitrogen-doped carbon dots (NCDs)

The nitrogen-doped carbon dots (NCDs) employed in this study were synthesized according to a reported method [23], which was modified according to our needs. Briefly, citric acid (0.42 g) and ethylenediamine (530 µL) were dissolved completely in 10 mL of ultrapure water to achieve a molar ratio of 1:4 (citric acid: ethylenediamine). The solution was prepared in a 50 mL Teflon autoclave liner, which was placed into a standard stainless steel 304 autoclave reactor (purchased from Labware on Amazon.com, part number: 2T50, Wilmington, DE, USA) and heated in an Isotemp oven (model 506G; Fisher Scientific, Waltham, MA, USA) at 185-195°C for 5 h. After allowing the reaction vessel to return to room temperature, the reaction mixture was centrifuged and decanted to remove any precipitate. A 5 mL aliquot of the neat reaction product solution (which was a clear, brownish-yellow solution) was dialyzed against ultrapure water for 3 days (while changing the water every 3 h, at room temperature) using a 100-500 Da MWCO cellulose ester dialysis membrane (Repligen Corp., Waltham, MA, USA). The dialysis membrane was conditioned according to the manufacturer's guidelines. The concentration of the dialyzed NCD solution thus produced was determined from representative aliquots: the reaction product solution was centrifuged and three, 0.5 mL aliquots were lyophilized with a benchtop freeze dryer (Labconco, Kansas City, MO, USA); and the average aliquot mass was then determined, to provide a mass-per-volume (mg/mL) concentration for the corresponding batch product solution. Typical “neat” solution concentrations for a batch of NCDs prepared according to the methods described herein were 50-60 mg/mL. Dialyzed, aqueous

NCD solutions were stored at room temperature in the dark until needed.

2.4. Instrumentation

All ctITP experiments were performed on a Sciex P/ACE MDQ plus CE system with LIF detection (employing an Ar-ion laser for excitation at 488 nm, with a 520 nm long pass filter and 488 notch filter for emission) (Framingham, MA, USA). Data were collected and analyzed using Sciex 32 Karat™ software. Uncoated, fused-silica capillary with inside diameter 20 µm (outside diameter, 365 µm) was obtained from Polymicro/Molex (Phoenix, AZ, USA), and was cut to length (40.0 cm effective length; 50.2 cm total length) for all ctITP experiments described herein. All samples were injected by pressure (2 psi for 9 s) directly onto the capillary, resulting in an injection volume of 1.0 nL. Electrokinetic separations were achieved by applying a constant voltage of 15 kV.

3. Results and Discussion

Free solution CE-based separation methods are not typically able to resolve DNA samples of varying length in the range of 10-150 bases [7]. However, given the ability of CDs to noncovalently interact with DNA molecules of varying length, we set out to examine the possible utility of CDs as buffer additives to facilitate such separations. The properties of CDs and the nature of their interactions with target analytes can be further tuned by the addition of dopants during synthesis, such as the addition of nitrogen during our CD synthesis from citric acid with ethylenediamine. The resulting NCDs yielded stronger fluorescence emission than their non-doped counterparts when excited by UV light (see Fig. 1A). This behavior is consistent with previously reported N-doping effects [24, 25]. Our NCDs demonstrated fluorescence excitation and emission maxima at 360 nm and 445 nm, respectively. Fluorescence emission intensity increased linearly with increasing NCD concentration (see Fig. 1B), up to a maximum of 500 μg/mL of NCDs. The NCDs were stable in aqueous solution at room temperature for at least 6 months. As determined by scanning electron microscopy, the NCD size was nominally 5-10 nm.

By employing these NCDs as additives in both the sample and separation buffers in ctITP, we capitalized on their interactions with DNA (occurring by way of π-π stacking, hydrophobic interactions, hydrogen bonding, and/or electrostatic interactions [14]) in order to effect a separation of ssDNA samples ranging in length from 32-100 bases, as seen in Fig. 2A. In particular, the preparation of nitrogen-doped carbon dots under hydrothermal conditions leads to the formation of pyrrolic N within the graphene framework [25], which may further enhance interactions with DNA compared to undoped CDs. Under similar experimental conditions but in the absence of carbon dots, ssDNA molecules of different length co-migrated and could not be resolved (as seen in Fig. 2B), due to the similar charge-to-size ratios possessed by DNA molecules in the 10-150 base range. Even the use of undoped CDs as ctITP buffer additives did not yield a separation of the ssDNA samples (as seen in the Supplementary Materials, Fig. S1).

Additionally, we conducted an experiment identical to that in Fig. 2A but with unlabeled versions of the ssDNA samples (and employing absorbance detection at 254 nm), to determine if the FAM label, in and of itself, might be responsible for the interactions between NCDs and DNA. Without the FAM label present, resolution of the three ssDNA samples and their order of migration (from first to last: 100-base, 61-base, 32-base) was unchanged (data not shown) relative to the FAM-labeled samples with NCDs employed in the ctITP system. Hence, we confirmed that the DNA-NCD interactions were not dependent upon the presence of the FAM label on the DNA.

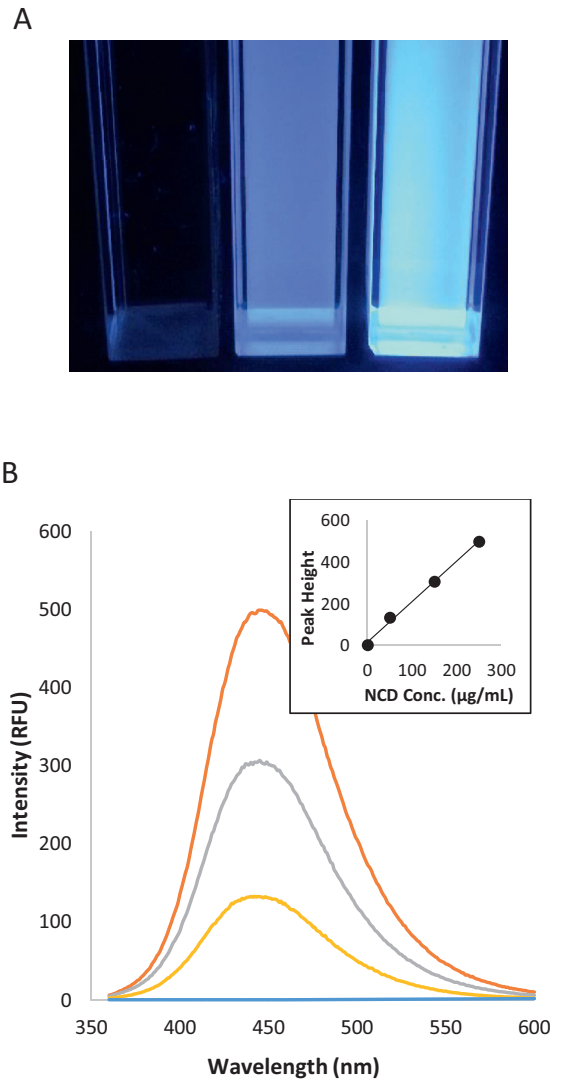

Fig. 1. (A) Photograph of carbon dot samples taken under illumination by a hand-held UV lamp (365 nm), showing water only (left), undoped citric acid CDs (center), and NCDs prepared from citric acid with ethylenediamine (right), at a concentration of 30 μg/mL. (B) Fluorescence emission spectra (λ_{excit} = 360 nm) for NCD samples in water (0 μg/mL, blue curve; 50 µg/mL, yellow curve; 100 μg/mL, grey curve; 250 µg/mL, orange curve). Inset: calibration curve for NCD emission intensity as a function of concentration: y=1.9481x+15.417; R^2=0.9955).

The three traces in Fig. 2.B represent three different sample concentrations (100 nM (i), 150 nM (ii), and 200 nM (iii) of each of the three sizes of DNA in a sample mixture). From these results, it can be seen that ctITP in the absence of CDs does not allow for the resolution of the three lengths of ssDNA in these samples, but it does allow for the quantification of total DNA concentration. That is, the single peak seen in each trace of Fig. 2.B, which represents the signal arising from the co-migrating 100-base, 61-base, and 32-base ssDNA samples, increases in area proportionately with DNA sample concentration (see Fig. S2-B, for the corresponding calibration curve). Similarly, the sum total of peak areas for the resolved ssDNA peaks in separations with NCDs (Fig. 2A) increases proportionately with total DNA concentration (see Fig. S2-A for the corresponding calibration curve).

While ctITP conducted in the absence of CDs (or, with undoped CDs) was unable to provide resolution of ssDNA of different lengths, it is notable that the same experiments conducted with nitrogen-doped CDs were able to achieve the desired separation.

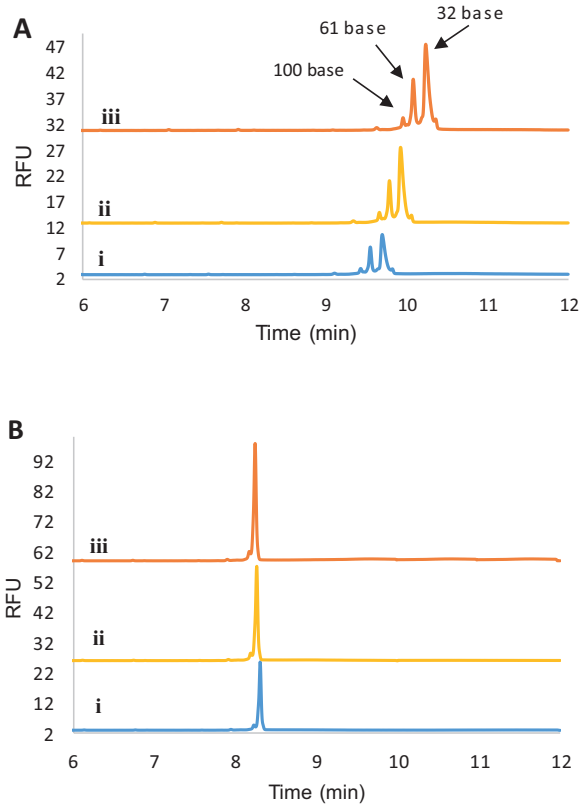

Fig 2. Vertically offset electropherograms of samples containing various sizes of ssDNA (100, 61, and 32 bases) prepared with (i, blue) 100 nM, (ii, yellow) 150 nM, or (iii, red) 200 nM of each of the three sizes of DNA: (A) with 30 μg/mL NCDs in the sample and separation buffers; (B) without added NCDs in the buffers. Buffers were: 15 mM tris/500 mM glycine at pH 7.92 (separation) and 50 mM tris adjusted to pH 8.0 by the addition of 1.0 M HCl (sample). Injection, separation, and detection conditions were as described in Section 2.

To our knowledge, such a separation has not been achieved by any other free-solution electrokinetic method. The ctITP separation of ssDNA samples enabled by the addition of 30 μg/mL NCDs to the separation and sample buffers alike is shown in Figure 2A. The different sizes of ssDNA samples were not only resolved by this NCD-modified ctITP method, but they were also retarded in their net migration towards the detector, as evidenced by the longer migration times in Fig. 2A compared to 2B. Perhaps it was this very fact (longer migration times under comparable separation conditions) that enabled the resolution of different lengths of ssDNA in the present work. However, a longer migration time alone would not lead to differentiation between the various lengths of ssDNA. Such resolution must be due to differential interactions of the NCDs with different lengths of ssDNA. The stronger the interaction between NCDs and DNA, the longer the migration time of the resulting complex. This is because the NCDs themselves have a negative electrophoretic mobility (towards the cathode/away from the detector) and so the association of any (DNA) sample ions with NCDs will result in a reduced net mobility for the sample.

Other carbon materials, such as graphene oxide (GO), have been shown to have different affinities for ssDNA samples of different lengths [15]. In this prior work by He et al., short ssDNA was shown to interact more strongly with GO than long ssDNA. As seen in Fig. 2.A, the relative order of migration of complexes of NCDs with ssDNA samples in our current work was (from fastest to slowest): 100 bases, followed by 61 bases, followed by 32 bases, which follows the trend predicted by He et al.'s GO studies. In our current work, a shorter (faster) migration time for the larger ssDNA molecules suggests these DNA molecules spend less time associated with the (negatively migrating) NCDs. The longer (slower) migration time of the smallest length of ssDNA (32 bases) in our work is indicative of greater interactions with the NCDs, and thus, greater retardation of the sample's mobility. We confirmed these differential interactions between NCDs with ssDNA samples of different lengths by conducting Stern-Volmer-type fluorescence quenching experiments (see Fig. S3), whereby shorter DNA samples were shown to have stronger associations with NCDs than longer DNA samples.

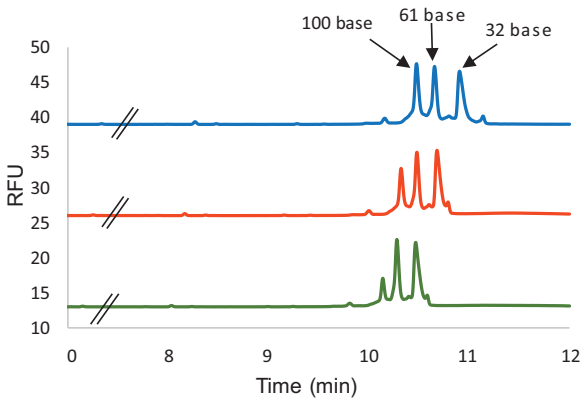

Fig. 3. Spiking experiments to identify and quantify 100-base ssDNA in a sample mixture consisting of: (i, green) 400:200:100 nM, (ii, red) 800:200:100 nM, and (iii, blue) 1200:200:100 nM of 100-base:61-base:32-base ssDNA. In each case, separation and sample buffers (as described in Fig. 2) contained 30 μg/mL NCDs. All other ctITP conditions were as described in Section 2.

Peak identities were confirmed by performing spiking experiments. These experiments also demonstrated linear relationships between individual ssDNA concentrations and peak areas in electropherograms employing NCDs for ssDNA resolution. For example, the concentrations of 61- and 32-base ssDNA samples were held constant (at 200 nM and 100 nM, respectively) in each of three sample mixtures, which also contained 100-base ssDNA at a concentration of 400 nM, 800 nM, or 1200 nM (see Fig. 3, traces i, ii, and iii, respectively). The increase in peak height and area for the first of the three major peaks in the resulting electropherograms confirmed this to be the peak corresponding to the 100-base ssDNA sample component. Similar spiking experiments (holding the concentrations of two of the three ssDNA samples constant while varying the concentration of the third, for 32-base and then 61-base samples; data not shown) confirmed the remaining peak identities, which are as labeled in Fig. 3. Linear calibration curves were obtained for each of the three ssDNA samples in the mixtures (as can be seen in Fig. S4); however, sensitivities (slopes of the calibration curves) for the three ssDNA samples differed. The FAM-labeled 100-base sample yielded the lowest sensitivity, followed by the labeled 61-base and 32-base samples. The practical implication of this is that equimolar concentrations of FAM-labeled ssDNA samples containing different numbers of bases yield different peak heights (and areas) by ctITP with LIF detection, and so quantitation by this method would require separate calibration curves.

The effect of NCDs on the mobility of ssDNA samples of different lengths and concentrations is quantified in Table 1, along with resolution values. First, it is noted that the electroosmotic mobility μ(eo) in the ctITP system with NCDs is only 55% of that without added carbon dots. This overall reduction in electroosmotic flow is likely due, in large part, to the high charge density on the NCDs themselves, which would contribute to a higher overall ionic strength buffer system, and thus, lower electroosmotic flow. Second, for a given concentration of DNA sample in the ctITP experiments with NCDs in the buffers, the absolute value of the electrophoretic mobility |μ(ep)| of each DNA peak increased as the

Table 1
Electroosmotic μ(eo), electrophoretic μ(ep), and net mobilities μ(net) for ssDNA samples of different lengths, with and without NCDs added to separation and sample buffers in ctITP. Resolution R_s values for adjacent peaks are reported in parentheses.

Conc. of ss-DNA (nM)	with NCDs μ(eo)/10^{-5} = 60.1 $cm^2V^{-1}s^{-1}$								without NCDs μ(eo)/10^{-5} = 109.3 $cm^2V^{-1}s^{-1}$					
	μ(net)/$10^{-5}cm^2V^{-1}s^{-1}$					μ(ep)/$10^{-5}cm^2V^{-1}s^{-1}$			μ(net)/$10^{-5}cm^2V^{-1}s^{-1}$			μ(ep)/$10^{-5}cm^2V^{-1}s^{-1}$		
	100 base		61 base	32 base		100 base	61 base	32 base	100 base	61 base	32 base	100 base	61 base	32 base
100	23.5	(R_s= 1.77)	23.3	(R_s= 1.30)	22.9	-36.5	-36.8	-37.2	27.4	27.4	27.4	-81.9	-81.9	-81.9
150	23.0	(R_s= 1.71)	22.7	(R_s= 1.28)	22.4	-37.1	-37.4	-37.7	27.6	27.6	27.6	-81.7	-81.7	-81.7
200	22.3	(R_s= 1.76)	22.0	(R_s= 1.33)	21.7	-37.8	-38.0	-38.4	27.7	27.7	27.7	-81.6	-81.6	-81.6

length of the DNA decreased (from 100 bases down to 32 bases long). As mentioned previously, this suggests that the shorter DNA samples are more strongly associated with the NCDs, and their resulting complexes experience greater retardation as a result. Third, the net mobility μ(net) of the DNA peaks decreased with decreasing length of DNA. This results in the appearance of the longest DNA first and the shortest DNA last in the electropherograms in Fig. 2A. Additionally, it can be seen from Table 1 that the absolute values of the electrophoretic mobilities $|\mu(ep)|$ of DNA samples increased and the net mobilities μ(net) decreased with increasing DNA concentration (from 100 nM of each of the three sizes of DNA up to 200 nM of each) in the presence of NCDs. One possible explanation for this could be greater DNA-DNA interactions at higher concentrations of DNA, which could result in larger (and more negatively charged) NCD-DNA assemblies, and hence, longer migration times. However, the exact mechanism leading to this change in mobility with ssDNA concentration has not yet been determined.

From Table 1, it can also be seen that the 100-base, 61-base, and 32-base samples had the same μ(ep) and same μ(net) values for a given concentration of DNA in the absence of NCDs. This was observed in practice as the co-migration of all DNA samples in Fig. 2B. For a given size and concentration of DNA sample (take, for example, 100-base ssDNA at 100 nM), the $|\mu(ep)|$ and μ(net) values were greater than for comparable samples in the presence of NCDs. This was observed in practice as faster migrating DNA peaks in Fig. 2B (no carbon dots) compared to Fig. 2A (with NCDs).

4. Conclusions

This study demonstrates a new application for nitrogen-doped carbon dots as buffer additives capable of effecting the separation of ssDNA samples of different lengths via a free solution ctITP method. DNA molecules with fewer bases showed a stronger affinity towards NCDs than their longer DNA counterparts. This differential interaction with the NCDs in the separation buffer during the electrokinetic separation impacted the mobilities of the DNA samples, thus resulting in their separation. Such a separation was not possible in the absence of NCDs, owing to the fact that DNA molecules in the 10-150 base range possess similar charge-to-size ratios and so are not separated by free solution CE methods. It was also found that undoped (citric acid) CDs did not provide for the resolution of 100-, 61-, and 32-base ssDNA samples under ctITP conditions equivalent to those employed with NCD-modified buffers.

NCDs are inexpensive and easy to prepare, and when employed at low concentrations (30 μg/mL, as in this work), they do not interfere with the fluorescence emission of covalently FAM-labeled DNA samples. As such, CE-LIF-based methods involving NCDs as buffer additives can yield quantitative results for analyte determination. While many electrokinetic-based separation protocols could potentially be improved by employing NCDs as buffer additives, it is likely that aptamer discovery studies and aptamer assays for biomolecular or small drug molecule targets could benefit most directly. Work in this area is continuing in our lab.

Author Contributions

D.R. conducted the experimental work including NCD synthesis and ctITP separations, as well as the initial data analysis and manuscript draft. C.L.C. provided oversight of the experimental design, along with contributing to final data analysis and manuscript writing. Data curation and funding acquisition were provided by C.L.C.

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CRediT Author Statement

Debashish Roy: Writing-original draft, Methodology, Visualization, Investigation, Formal Analysis; **Christa L. Colyer:** Writing-review and editing, Supervision, Project administration, Funding acquisition, Resources, Conceptualization, Data curation, Methodology

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.461990.

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