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Analysis of lipid-oligonucleotide conjugates by cyclodextrin-modified capillary zone electrophoresis

Fatima Barakat¹, Karen Gaudin^{1,*}, Brune Vialet¹, Katell Bathany², Sebastien Benizri¹, Philippe Barthélémy^{1,*}, Ludivine Ferey^{1,*}

¹ ARNA INSERM U1212, CNRS 5320, University of Bordeaux, Bordeaux, F-33076, France

² Chimie et Biologie des Membranes et Nanoobjets (CBMN) UMR 5248 CNRS, Université de Bordeaux, 33600 Pessac, France

*email: ludivine.ferey@u-bordeaux.fr

*email: karen.gaudin@u-bordeaux.fr

*email: philippe.barthelemy@inserm.fr

ABSTRACT

Lipid-oligonucleotide (LONs) based bioconjugates represent an emerging class of therapeutic agents, allowing the delivery of therapeutic oligonucleotide sequences. The LON development requests accurate and efficient analytical methods. In this contribution, LON analysis methods were developed in cyclodextrin-modified capillary zone electrophoresis (CD-CZE). The LONs selected in this study feature different structures, including i) the oligonucleotide length (from 10 to 20 nucleotides), ii) the inter-nucleotide linkage chemistry (phosphodiester PDE or phosphorothioate PTO), and iii) the lipidic part: single- (LONsc) or double- chain (LONdc) lipids. In CD-CZE, the effect of several parameters on the electrophoretic peaks was investigated (buffer, CD, and capillary temperature). The binding interaction between LON and Me- β -CD was studied in affinity capillary electrophoresis and revealed a 1:1 LON:CD complex. Non-linear regression and three usual linearization methods (y-reciprocal, x-reciprocal, and double-reciprocal) were used to determine the binding constants (K values of $2.5 \cdot 10^4 \text{ M}^{-1}$ and $2.0 \cdot 10^4 \text{ M}^{-1}$ for LON_{PDE} and LON_{PTO}, respectively). Quantitative methods with good performances and analysis time lower than 5 min were achieved. Importantly, the developed analysis allows a separation between the i) full-length sequence LONs and their truncated sequences, (n-1), (n-2), and (n-4)-mers and ii) LONsc, LONdc and their corresponding unconjugated oligonucleotides. This work highlights the interest of CD-CZE methods for LON analysis.

Key words: capillary electrophoresis, lipid-oligonucleotides, bioconjugates, oligonucleotides, cyclodextrin, binding constant

1. Introduction

Oligonucleotide-based therapeutics are chemically synthesized oligonucleotides featuring a single-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) structure with potential specific interaction. Thanks to this specificity, these derivatives can interfere with gene expression or inhibit protein function *via* different mechanisms, including antisenses (ASOs), microRNAs (miRNAs), small interfering RNA (siRNA), or aptamers [1]. Therapeutic developments of these molecules take advantages of a constant evolution of nucleic acid chemistry, which opens new opportunities for numerous diseases. Hence, a wide range of chemical modifications have been developed to address challenges in the field, including the delivery of active sequences.

The bioconjugation of oligonucleotides offers new opportunities at the chemical-biology interface and is currently meeting increasing success for clinical applications [2]. These conjugates have been investigated to allow tissue targeting or improve cellular uptake for example. Among them, lipid

oligonucleotide conjugates [3] have been reported for different biomedical applications [4–6], to inhibit the hepatitis C virus translation [7], for improving cellular uptake of therapeutic antisenses [8] or for tackling the antibiotic resistance [9]. Thanks to their amphiphilic character, these lipid-oligonucleotides (LONs) offer unique self-assembly properties. They have been used either as functional reservoir for lipophilic drugs [3,10] or as a vectorization system of the antisense sequence avoiding the use of transfecting agent [7–9].

To support LON pharmaceutical developments, new analytical methods are needed to properly quantify LONs in formulations but also to test the purity of synthesized products. Indeed, similarly to unconjugated oligonucleotides (ON), LONs are produced using an automatic synthesizer through a solid-phase synthesis which generates structurally closely related impurities, making efficient separation methods a key challenge for the future development of LON bioconjugates.

Until now, separation methods of choice for unconjugated oligonucleotides analysis have been liquid chromatography using ion-pairing [11–16], ion-exchange modes [11,17,18], and gel electrophoresis [19–21]. Therapeutic oligonucleotides have also been investigated using mass spectrometry [22].

Compared to non-conjugated oligonucleotides, the analysis of LONs appears particularly challenging due to their low solubility in water, leading to detrimental adsorption onto silica surfaces (chromatographic columns or silica capillaries) and their amphiphilic nature (coming from the apolar long-chain fatty acid and the polar oligonucleotide sequence), resulting in self-aggregation phenomenon.

In literature, to our knowledge, there is no publication dealing with the quantitative analysis of LONs or structurally related compounds. The only article we found deals with the identification of the major impurities of two phosphorothioate (PTO) oligonucleotides conjugated to a single-chain lipid by ion-pair reversed-phase HPLC combined with MALDI-TOF-MS [23]. Compared to HPLC, capillary electrophoresis (CE) offers several advantages such as fast analysis, high efficiency of peaks, and low consumption of samples, reagents, and solvents.

In this study, we investigate a novel approach involving cyclodextrin-modified capillary zone electrophoresis (CD-CZE) for LON analysis. A comprehensive study was carried out by varying the chemical structures of LONs and the electrolyte composition. For the first time, fast and efficient analysis methods were achieved in CE for phosphodiester (PDE) and PTO based LONs and their respective non-conjugated ONs. In addition, the interaction between LONs and Me- β -CD was further studied using affinity capillary electrophoresis (ACE) through the determination of complex stoichiometry and binding constants.

2. Materials and methods

2.1 Chemicals and buffer preparation

All chemical and solvents used were of analytical grade or of HPLC quality.

β -CD was supplied by Sigma (Saint-Louis, MO, USA). α -CD, γ -CD, and dimethyl- β -CD (Me- β -CD) were purchased from Acros Organics (Fisher Scientific, Illkirch, France) and 2-hydroxypropyl- β -CD (HP- β -CD) was from Cyclo Lab LTD (Budapest, Hungary). The tetraborate sodium buffer (10 mM, pH = 9.5) was prepared from $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (VWR, Fontenay-sous-Bois, France). Different concentrations of CDs were prepared with this tetraborate buffer for ACE experiments. The phosphate buffer (40 mM, pH = 7.0) was prepared by mixing appropriate amounts of NaH_2PO_4 (Carlo Erba, Val-de-Reuil, France) and Na_2HPO_4 (Sigma, Saint-Louis, MO, USA).

A thiourea solution at 0.01% (w/w) in water was used as an electroosmotic flow (EOF) marker. Thiourea was from VWR (Fontenay-sous-Bois, France).

A MilliQ RG water purification system (Millipore, Bedford, MA, USA) was used to provide ultrapure water (18.2 MΩ.cm).

All buffers were filtered through a 0.20 μm nylon membrane from VWR supplier.

2.2 LON synthesis and samples

LONs were synthesized using the conventional phosphoramidite chemistry on an automated H8 synthesizer (K&A Laborgeraete, Germany) at the μmole scale. The oligonucleotide sequence (5' TGC TCA CAA CAG GC 3') was chosen to minimize the chances of forming undesired secondary structures due to its low number of guanines and the short length of its sequence, as described in previous studies [24]. Oligonucleotides were modified at the 5'-end with two different lipid phosphoramidites: a simple chain C18 lipid (LONsc) or a ketal double chain C16 nucleolipid (2',3'-O-16-hentriacontanylidene-uridine) (LONdc). Both phosphoramidites were synthesized according to literature procedures [24]. LONs were synthesized using PDE or PTO chemistry for the internucleotide link. Corresponding unconjugated oligonucleotides were synthesized as controls. Truncated sequences (n-1), (n-2) and (n-4)-mers at the 5'-end for LONdc PDE and a 20-mers LONdc PTO (5' AAC TTG TTT CCT GCA GGT GA 3') [8] were also synthesized for selectivity assessment.

The purification of LONs was performed in reversed-phase HPLC on a Waters System with gradient standard protocol. The Xbridge protein BEH C4 column (30 x 50 mm, 5 μm, 300 Å) from Waters was used. A solution containing 100 mM triethylammonium acetate (TEAA) in 95:5 (v/v), water/acetonitrile (ACN) at pH 7.0 was used as solvent A and solvent B contained 20 mM TEAA in 20:80 (v/v), water/ACN. A linear gradient was programmed from 100% A to 100% B in 2 min. A mobile phase composed of 100% B was kept during 1 min. The column was returned to 100% A in 0.2 min and then equilibrated at 100% A for 0.8 min. The flow rate was fixed at 56.0 mL/min. The injection volume of samples was 5 mL. LONs were detected at a wavelength of 260 nm.

All compounds were characterized by Mass Spectrometry. Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry analyses were performed using a MALDI-ToF-ToF Ultraflex III (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam™ laser technology. Average molecular masses of the products were measured in the linear positive mode and a mixture of standard compounds was used for external calibration in the suitable mass range (3-6kDa). For each sample, a MALDI mass spectrum was acquired by accumulating the ion signal from 1000 laser shots and the data analysis was performed by FlexAnalysis.

LONs were dissolved in deionized water to a concentration of 20 μmol/L. The matrix 2,4,6-trihydroxyacetophenone (THAP, Sigma-Aldrich) was prepared at a concentration of 20 mg/mL in a 4:1 mixture of ethanol and 100 mM aqueous ammonium citrate. A drop (1 μL) of analyte/matrix (1:1) mixture was deposited on the MALDI target plate and allowed to dry at room temperature before analysis. For all compounds, theoretical and experimental average molecular masses and MALDI Mass Spectrum are presented in Supplementary Data (Table S1, Figures S1, S2, and S3).

The LON concentration in samples was determined with a NanoDrop One UV-Vis spectrophotometer (ThermoScientific) based on absorbance measurement at 260 nm with 2 μL of sample. The concentration was calculated according to the molecular absorption coefficient of each molecule.

2.3 Softwares

Non-linear and linear regressions of the experimental data were performed using Kaleidagraph and Excel softwares. The OligoAnalyzer Tool available on the Integrated DNA Technologies (IDT) website (<https://www.idtdna.com>) was used to determine the molecular absorption coefficient of each oligonucleotide at 260 nm.

2.4 Apparatus and electrophoretic procedures

CE experiments were performed on a Beckman Coulter P/ACE MDQ system hyphenated with a UV detector (Sciex, Framingham, MA). Data were collected using 32Karat software (Beckman Coulter, Sciex, Framingham, MA).

The CE system was equipped with a capillary cartridge filled with a liquid coolant allowing efficient temperature control. Uncoated fused-silica capillaries (50 μm i.d. x 375 μm o.d.) (Photon Lines SAS, Saint-Germain-en-Laye, France) with effective and total lengths of 30 cm and 40.2 cm, respectively, were used.

A new capillary was conditioned by successive rinsing with 1 M NaOH (20 psi, 3 min), 0.1 M NaOH (20 psi, 3 min), ultra-pure water (20 psi, 3 min), and finally background electrolyte (BGE) (20 psi, 30 min). Between each run the capillary was rinsed with the BGE (20 psi, 5 min). At the end of the day, the capillary was flushed with water (20 psi, 5 min).

LON samples were injected hydrodynamically under 0.5 psi for 10.0 s at the anodic side of the capillary. Analyses were performed in triplicate. A voltage of +20 kV was applied and different capillary temperatures were tested between 15°C and 45°C, corresponding to the limits of the CE system. Detection was carried out at 254 nm.

2.5 Analytical performances

Different criteria were studied to assess method performances: linearity, limits of detection (LOD) and quantitation (LOQ), precision, and accuracy.

Linearity of the method was tested at five different concentration levels (Table S2) of LONs in purified water. The calibration curves were obtained using the least-square linear regression by plotting the corrected peak area versus LON concentration.

LOQ and LOD values were calculated from the calibration curves and corresponded to LON concentrations for which response values were equal to 10 times and 3.3 times of the standard deviation of the slope, respectively.

Precision was evaluated through the repeatability (intra-day) and the intermediate precision (inter-day) by injecting each molecule at 10 μM in water. Precision was expressed as relative standard deviation (%RSD) values for corrected peak areas. Repeatability was assessed from 3 replicates performed during the same day (intra-day), while the intermediate precision was investigated by repeating the analysis on 3 different days (inter-day).

The accuracy of the method was examined at a concentration of 10 μM by determining the recovery between the concentration measured by Nanodrop and the one calculated from the CE calibration curve.

3. Theory for binding constant determination by affinity capillary electrophoresis (ACE)

After injection, the high dilution of LON samples below the micromolar concentration was supposed to prevent aggregation effect. Consequently, the hypothesis that LON only exist as monomers for interacting with CDs was made and a 1:1 stoichiometry model was considered.

At equilibrium, for a classical 1:1 binding interaction, the binding constant, K , is described as in Eq.1

$$K = \frac{[\text{LON:CD}]}{[\text{LON}][\text{CD}]} \quad (\text{Eq.1})$$

Where [LON], [CD], and [LON:CD] are the concentrations at equilibrium of free LON, free CD, and LON-CD complex, respectively.

Binding constants were calculated using ACE based on a change in LON electrophoretic mobility when CDs are added to the BGE due to a complexation phenomenon. The K values were determined by injecting a constant quantity of LON while increasing the CD concentration in the BGE. In that

case, the LON effective electrophoretic mobility (μ_i) depends on the fraction of time it spends attached to the CDs (f_{CD}), the free LON electrophoretic mobility in the absence of CD (μ_f), and the LON:CD complex electrophoretic mobility (μ_c):

$$\mu_i = \frac{1}{1+K[CD]} \mu_f + \frac{K[CD]}{1+K[CD]} \mu_c \quad (\text{Eq.2})$$

$$f_{CD} = \frac{K [CD]}{1+K[CD]} \quad (\text{Eq.3})$$

[CD] is the free CD concentration at equilibrium which can be approximated to the total CD concentration added to the buffer since it is much higher than the analyte concentration [25,26].

To obtain accurate values of binding constants, the shift in LON electrophoretic mobility observed in the presence of CDs must be only due to the complexation between LONs and CDs. However, the viscosity of the BGE is increased when CDs are added, modifying electrophoretic mobility of analytes. Consequently, the impact of the CD concentration on BGE viscosity and consequently, on LON electrophoretic mobility must be assessed and proved to be negligible or otherwise must be taken into account.

To calculate binding constants, non-linear regressions and three different linear transformations called double-reciprocal, y-reciprocal, and x-reciprocal equations are commonly used. Non-linear regressions have been proved to be better in terms of accuracy because experimental error on parameters is distorted when using linear regressions [27]. For each regression method, mathematical equations, plotting methods, and the calculation of binding constants (K) and LON:CD complex electrophoretic mobility (μ_c) are gathered in Table S3.

4. Results and discussion

In the literature, classical BGEs used for the analysis of non-lipidic ONs in CZE are composed of borate, acetate, or carbonate adjusted to a pH between 9.3 and 9.7 [28,29]. Therefore, first analyses of single-(LONsc) and double-chain (LONdc) LON PDE and PTO and their corresponding non-conjugated ONs used as control, were carried out using a 40 mM borate buffer (pH = 9.5). In addition, LONs were injected at a low concentration of 10 μ M in samples while capillary and sample temperatures were fixed as high as 45°C to prevent self-assembly phenomenon that can occur through lipophilic interactions between LON lipidic chains.

Good electrophoretic profiles were obtained for non-conjugated ONs whereas high peak tailing and asymmetry values were observed for LONs. Moreover, poorer peak shape was noticed for LONdc compared to LONsc. Such peak distortions noticed for LONdc were probably due to their lipidic moiety which may cause solubility problems and/or adsorption phenomenon onto the capillary walls. Also, even if experimental conditions were chosen to minimize LON self-assembly, some aggregates could exist in solution. Such aggregation phenomenon would be even more enhanced for double-chain LONs.

Also, whatever the inter-nucleotide linkage chemistry (PDE or PTO) or the nature of the lipidic part (LONsc or LONdc), no difference was observed in the migration times showing a low selectivity of analysis.

4.1 Effect of type and concentration of CD

To address the previous limitations, CDs were added to the BGE. CDs are frequently used in CE as chiral selector for enantioseparation or to improve the solubility and the selectivity of analysis of hydrophobic compounds through complexation. CDs are cyclic oligosaccharides composed of α -1,4 linked D-glucopyranose units arranged in a toroidal form. Owing to their hydrophobic cavity, CDs are able to form inclusion complexes with molecules possessing a hydrophobic group that fits the CD cavity in terms of size and shape. Adding different chemical modifications on the CD ring may change its solubility, the size of its cavity, and its hydrophobicity, thus modifying the interactions developed with the guest molecules [30].

For LON analysis, the addition of CDs to the BGE clearly improved peak shape (Figure S4). This could be explained by an increase in LON solubility in the aqueous BGE and/or a destabilization of LON assemblies due to complexation with CDs. In this work, CDs with different cavity sizes (α -CD, β -CD, and γ -CDs) and chemical substitutions (β -CD, Me- β -CD, and HP- β -CD) were studied at a constant concentration of 15 mM.

In the presence of CDs in the BGE, the effective electrophoretic mobility (μ_{ep}) observed for LONs depends on two parameters, the fraction of time the LON spends attached to the CD (f_{CD}), and its mobility when attached to a CD (μ_c) as expressed in equations 2 and 3. Consequently, the electrophoretic mobility of LONs becomes less negative upon increasing the strength of partitioning to the CDs.

As highlighted in Figure 1, the type of CD had a low impact on the electrophoretic mobility (μ_{ep}) values of LONs. LON electrophoretic mobilities in absolute values ($|\mu_{ep}|$) were slightly lower using Me- β -CD, especially for LONdc, showing a better affinity for Me- β -CD compared to other CDs. On the contrary, LONs interacted to a lower extent with γ - and HP- β -CDs, as evidenced by higher $|\mu_{ep}|$ values. Otherwise, intermediate $|\mu_{ep}|$ values were calculated in the presence of α - and native β -CDs. Whatever the CD, μ_{ep} values of oligonucleotides followed the order: $|\mu_{ep}(\text{LONdc})| < |\mu_{ep}(\text{LONsc})| < |\mu_{ep}(\text{ON})|$ that could be explained by both the size of the molecule and the strength of its interaction with the CD. Among the different CDs, selectivity values were found similar (around 1.2 between ON and LONsc and 1.1 between LONsc and LONdc) highlighting the negligible effect of the CD on the electrophoretic separation of oligonucleotides. However, best resolutions between compounds were achieved using Me- β -CD, which was retained for further studies.

Using 10 mM Me- β -CD in the BGE, baseline electrophoretic separations of ON, LONsc, and LONdc were achieved in less than 5 min, as displayed in Figure 2. The order of migration ($t_m(\text{LONdc}) < t_m(\text{LONsc}) < t_m(\text{ON})$) followed the order of μ_{ep} values: the lower μ_{ep} in absolute value, the higher the global migration velocity leading to lower migration time. As already mentioned above, the addition of CDs to the BGE improved significantly the electrophoretic profile, leading to thinner peaks with calculated efficiency values of around 1.8×10^4 theoretical plates for LONdc PDE and PTO, 3.0×10^4 theoretical plates for LONsc PDE and PTO, and 7.0×10^4 theoretical plates for ON PDE. Lower peak efficiency was observed for ON PTO (down to 0.7×10^4 theoretical plates), coming from the many diastereoisomers existing in mixture due to the chirality of PTO groups. However, the temperature of analysis was found to have a beneficial impact on ON PTO peak with efficiency value rising to 1.2×10^4 theoretical plates at 45°C. Baseline resolutions between lipidic and non-lipidic ONs were achieved with Rs values ranging from 4.9 and 10.5 for LONdc/ON peak pair PDE and PTO, respectively and from 8.5 and 3.4 for LONsc/ON peak pair PDE and PTO, respectively. This method could therefore be used to assess the coupling efficiency after synthesis.

The impact of Me- β -CD concentration added to the running buffer was first studied on a wide range between 0 and 20 mM. As presented in Figure S5, when increasing Me- β -CD concentration from 0 to 1 mM, $|\mu_{ep}|$ values of LONs decreased abruptly due to their partitioning into CDs. In fact, when more host molecules are added into the BGE, the fraction of the bound LONs (f_{cd}) is higher and therefore LON electrophoretic mobility becomes less negative. Such mobility shift of LONs is also due to the increase in viscosity of the running buffer when increasing the CD concentration but to a negligible extent as mentioned below (see part 4.5 construction of binding isotherms). This decrease in $|\mu_{ep}|$ values was found sharper for LONdc compared to LONsc, emphasizing a stronger binding of LONdc to CDs. Above 1 mM, $|\mu_{ep}|$ values stabilized due to a total inclusion of LON molecules into CD apolar cavities. On the contrary, ON electrophoretic mobilities were almost not affected by Me- β -CD concentration over the whole range 0-20 mM, evidencing an absence of interaction between non-lipidic ONs and CDs. The slight decrease in $|\mu_{ep}|$ values of ONs was due to the increasing viscosity effect. The same tendency was observed for both PDE and PTO inter-nucleotide linkage chemistries.

4.2 Analysis of LON truncated sequences

Truncated sequences are shorter ONs, missing one or more nucleotides. They belong to the main impurities of synthesis of ONs. The (n-1)-mers are found at higher levels compared to the (n-2)-mers and shorter sequences which are generally encountered at low levels [12,19].

These impurities are also produced during LON synthesis. For quality control purposes, methods allowing the separation between the targeted LON and its truncated sequences are therefore necessary. In this work, the separation between the full length sequence LONdc PDE and three truncated sequences: (n-1), (n-2), and (n-4) was investigated in CD-CZE.

The impact of Me- β -CD concentration and capillary temperature were studied on the selectivity of analysis. At a constant temperature of 25°C, the separation quality was not modified when varying the Me- β -CD concentration between 10 and 20 mM. In fact, as evidenced previously, LONs were totally included into CD apolar cavities over this range of concentrations. Moreover, the deletion of one nucleotide did not sufficiently modify the charge over size ratio of LON:CD complex to be able to separate LON truncated sequences. On the contrary, the capillary temperature turned out to be a critical factor. A decrease of capillary temperature improved the separation between the different sequence lengths (from 10 to 14 nucleotides) for LONdc PDE, as evidenced in Figure 3. It can be explained by an increase of BGE viscosity, leading to lower migration velocities and thus better separation. At 15°C, a resolution of 1.4 was obtained between the full-length sequence LON and its (n-1)-mers.

In addition, Figure S6 reveals a linear increase of $|\mu_{ep}|$ with the number of nucleotides (between 10 to 20 nucleotides) for LONdc PDE ($y = 0.472x + 12.189$; $R^2 = 0.9933$). The electrophoretic behavior of LONs appeared homogenous in this range and therefore could be predictable.

The same trend was observed for the analysis of non-lipidic ON PDE and its truncated sequences. However, the separation between the different sequence lengths for ONs was found to be similar in absence of CD since ONs did not interact with CD, as discussed previously (data not shown).

4.3 Effect of pH and nature of buffer

Acido-basic properties of oligonucleotides need to be considered when studying the impact of the pH of the electrolyte on LON analysis. The pKa value of the phosphodiester group is around 2, meaning that the studied oligonucleotide sequence contains 14 negative charges at a pH above 4. Moreover, nucleic acids have ionizable nitrogenous bases each characterized by pKa values. Cytosine and guanine have two pKa values: 4.2 and 9.3 for cytosine and 2.1 and 9.3 for guanine. While thymine and adenine have one pKa of 9.3 and 3.5, respectively.

Using a 40 mM phosphate buffer, the pH of the BGE was tested at 3, 7 and 9. At pH 3, LONs could not be detected and ONs migrated at high migration times above 20 min under reversed polarity. At acidic pH, except thymines, all bases are partially protonated leading to a lower net negative charge, and thus a lower electrophoretic mobility. At higher pH values (7 and 9) and under normal polarity, ON and LON peaks were detected at low migration times with good peak shape. $|\mu_{ep}|$ values were substantially higher at pH 9 compared to pH 7 due to a partial ionization of guanine, cytosine, and thymine bases at pH 9 resulting to a more negatively charged oligonucleotide sequence. However, the effective selectivity calculated as the ratio between electrophoretic mobilities of species was not impacted by the pH. Dealing with the type of BGE (phosphate or tetraborate buffer), similar electrophoretic mobility values and peak shape were found.

4.4 Method performances

Method validation was performed using a BGE composed of sodium tetraborate (10 mM; pH = 9.5) and 10 mM Me- β -CD with capillary and sample temperatures set at 25°C.

Specificity of the method was demonstrated by its capacity to separate LONs with different structures (nature of lipid and oligonucleotide length).

Table 1 summarizes the results of method validation. The linearity of the method was assessed by the coefficients of determination (R^2) of the calibration curves obtained from least-square linear regressions and composed of five concentration levels. In the studied range of concentrations, good linear calibrations were obtained for all oligonucleotides with $R^2 > 0.98$. RSD values on corrected peak areas were below 2% and 7%, for intra-day and inter-day precisions, respectively. Recoveries were all above 89.6% (except for ON PTO) which confirmed the accuracy of the method. Poorer analysis performance was noticed for ON PTO because of low peak efficiency. LOQ and LOD values were below 9 μ M and 3 μ M, respectively.

4.5 Construction of binding isotherms

For a better characterization of LON:CD complexes, binding constants (K) and complex electrophoretic mobilities (μ_c) were determined by ACE. This study was carried out on the range 0 mM and 0.6 mM of Me- β -CD over which the complexation phenomenon occurred with LON, as previously described.

The impact of CD addition on buffer viscosity and consequently, on LON electrophoretic mobilities was first studied through the determination of viscosity correction factors, v , using Poiseuille's equation [31].

$$v = \frac{\eta}{\eta^0} = \frac{t}{t^0} \quad (\text{Eq.4})$$

Where η and η^0 are buffer viscosities with and without CD, respectively, and t and t^0 , the times required for thiourea to migrate from the injection end to the detector with and without CD, respectively. Over the CD concentration range investigated, viscosity correction factors fluctuated from 1.00 to 1.05. Consequently, the influence of an addition of CDs to the BGE was assumed negligible on LON electrophoretic mobilities. No correction of LON electrophoretic mobilities by v was therefore applied in this study.

Figure 4 presents the non-linear regressions of LON electrophoretic mobility difference in presence (μ_i) and in absence of CD (μ_f) versus Me- β -CD concentration. When CD concentration increased from 0.05 mM to 0.6 mM, LON μ_{ep} became less negative compared to μ_{ep} in absence of CD, as more LONs are complexed with CDs. Hence, the difference ($\mu_i - \mu_f$) increased with CD concentration. The data points were in good agreement with the theoretical binding isotherms with R^2 at least 0.991 (Table S4).

High correlation coefficients are not sufficient to prove that 1:1 model is the best model to describe data, consequently, the possibility of higher order interactions was investigated for LONdc. Indeed, the two lipidic chains of LONdc could form inclusion complexes with Me- β -CD. A model taking into account both 1:1 and 1:2 stoichiometries was therefore tested to fit the mobility data obtained to the following non-linear regression model:

$$\mu_i - \mu_f = \frac{(\mu_{c1} - \mu_f)K_{1:1}[CD] + (\mu_{c2} - \mu_f)K_{1:1}K_{1:2}[CD]^2}{1 + K_{1:1}[CD] + K_{1:1}K_{1:2}[CD]^2} \quad (\text{Eq.5})$$

where $K_{1:1}$, $K_{1:2}$, and μ_{c1} , μ_{c2} are the binding constants and the electrophoretic mobilities of complexes corresponding to 1:1 or 1:2 stoichiometric interactions, respectively.

This model gave a noticeably worse fit to the experimental data points as highlighted by lower R^2 (around 0.96) and inconsistent binding constants ($K_{1:1}$ values of 4735 M^{-1} and 13232 M^{-1} and $K_{1:2}$ values of 7238 M^{-1} and 214 M^{-1} for LONdc PDE and PTO, respectively). An inconsistency was also observed for electrophoretic mobilities of LON:CD complexes with $|\mu_{c2}|$ values higher than $|\mu_{c1}|$ values and μ_{c2} values much different between LONs (μ_{c1} values of $-14.2 \cdot 10^{-5} \text{ cm}^2 \cdot V^{-1} \cdot s^{-1}$ and $-14.4 \cdot 10^{-5} \text{ cm}^2 \cdot V^{-1} \cdot s^{-1}$ and μ_{c2} values of $-29.4 \cdot 10^{-5} \text{ cm}^2 \cdot V^{-1} \cdot s^{-1}$ and $-35.7 \cdot 10^{-5} \text{ cm}^2 \cdot V^{-1} \cdot s^{-1}$ for LONdc PDE and PTO, respectively). For all these reasons, the model mixing both 1:1 and 1:2 stoichiometries was definitely rejected.

Assuming a 1:1 interaction for LON:CD complexes, data were fit to three linearized forms: double-reciprocal, y-reciprocal and x-reciprocal as shown in Figure S7. A quite good linearity of the plots was observed ($R^2 > 0.913$). K , μ_c , and R^2 values obtained for both linear and non-linear regressions are gathered in Table S4. For each species, consistent K and μ_c values were obtained between the non-linear and the 3 linear methods. Binding constants of about $2.5 \cdot 10^4 M^{-1}$ and $2.0 \cdot 10^4 M^{-1}$ were

calculated for LON PDE and PTO, respectively, proving a better affinity of LON PDE for Me- β -CD compared to LON PTO. In addition, K values were found similar between LONsc and LONdc, demonstrating that the number of lipidic chains had no impact on the binding affinity. It is important to note that K values calculated in this study were of the same order of magnitude as those determined by Parker et al [32] for dodecanoate, the closest structural compound to LONs that could be found in the literature (K values of $1.4 \cdot 10^4 \text{ M}^{-1}$ and $2.6 \cdot 10^4 \text{ M}^{-1}$ obtained in ACE and isothermal titration calorimetry, respectively). The different regression methods led also to consistent electrophoretic mobility values for LON:CD complexes (μ_c values around $-29.0 \cdot 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ and $-26.0 \cdot 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ for LONsc and LONdc, respectively). $|\mu_c|$ values were lower for LONdc in comparison with LONsc due to the larger size of the complex involving a double-chain LON.

5. Conclusion

In this study, a new method using cyclodextrin-modified capillary zone electrophoresis was developed for the analysis of lipid-oligonucleotide (LON) based bioconjugates.

The results show that LON difficulty of analysis (coming from their adsorption onto silica surfaces and their ability to self-aggregate) was overcome in CD-CZE. LONs with different chemical modifications, including the oligonucleotide length (between 10 and 20 nucleotides), the inter-nucleotide linkage chemistry (phosphodiester or phosphorothioate), and the lipidic part (single- or double- lipid chains) were successfully analyzed in CD-CZE. The addition of a neutral CD to the sodium tetraborate buffer allowed an improvement of LON peak shape.

The CD-CZE method was proved to be quantitative (between 5 and 30 μM) with good intra-day ($\text{RSD} \leq 2\%$, $n=3$) and inter-day ($\text{RSD} \leq 7\%$, $n=3$) precisions on corrected peak areas and LOQ values below 9 μM . Moreover, the selectivity of analysis allowed a baseline separation in less than 5 min between the full-length sequence LONs and their truncated sequences (n-1), (n-2), and (n-4)-mers, and between LONsc, LONdc, and their corresponding unconjugated oligonucleotides.

The non-covalent binding interactions between LONs and CDs were studied in ACE. It was evidenced that the complexation between LONs and Me- β -CD involved a 1:1 association, whatever the number of lipidic chains. The binding constants were found similar for LONsc and LONdc and slightly higher for LON PDE compared to LON PTO ($2.5 \cdot 10^4 \text{ M}^{-1}$ and $2.0 \cdot 10^4 \text{ M}^{-1}$ for LON PDE and PTO, respectively). These results demonstrate that only one fatty acid chain interacts with the apolar cavity of the CD. Moreover, it can be concluded that LONsc and LONdc could be separated based on differences in LON:CD complex electrophoretic mobilities (around $-29.0 \cdot 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ and $-26.0 \cdot 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ for LONsc and LONdc, respectively), coming from the larger hydrodynamic radius of LON:CD complexes involving LONdc compared to LONsc.

Thanks to its selectivity and quantification capacity, this approach represents a promising analytical tool for the future pharmaceutical development of these new therapeutic agents.

6. References

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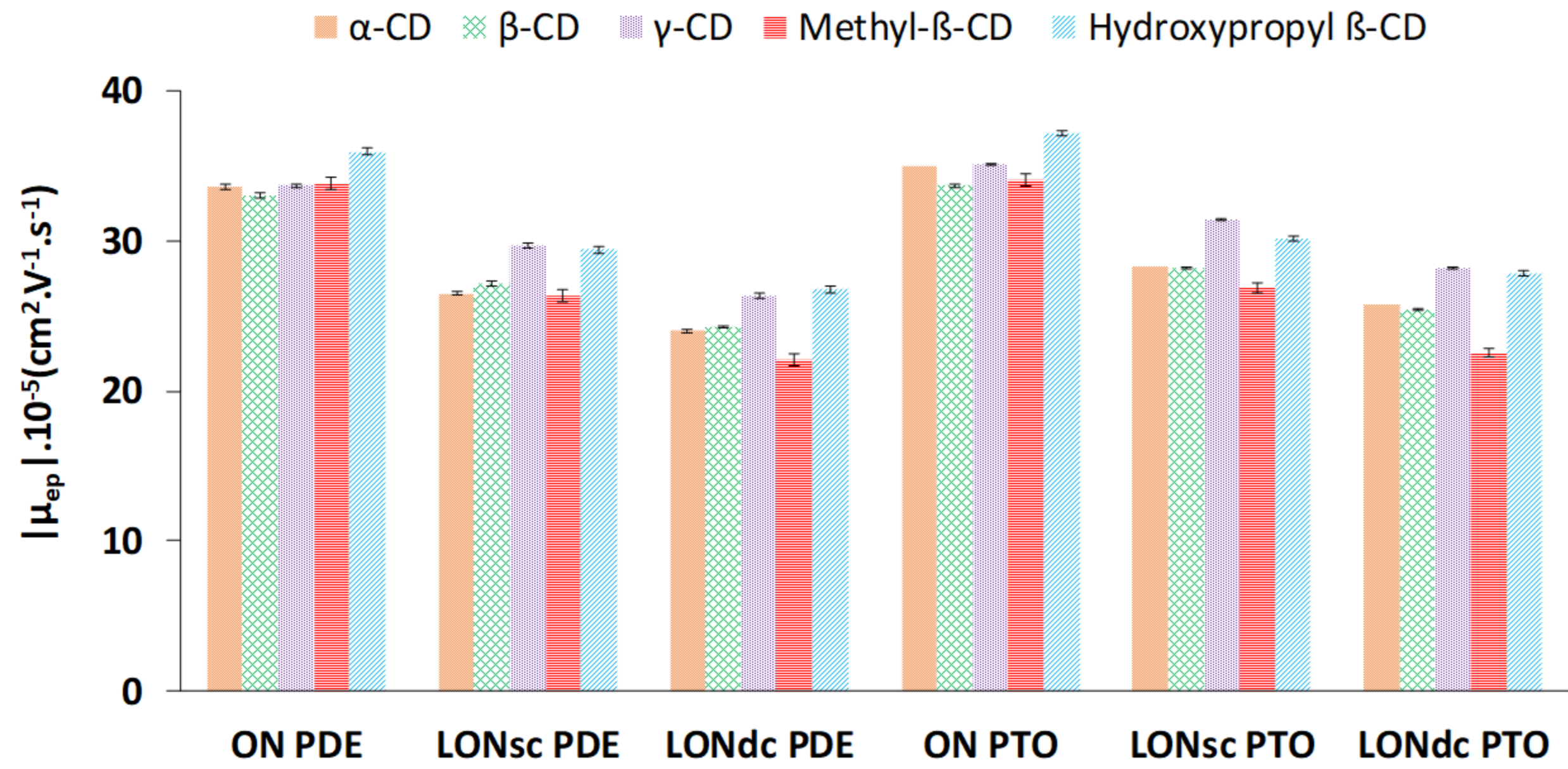
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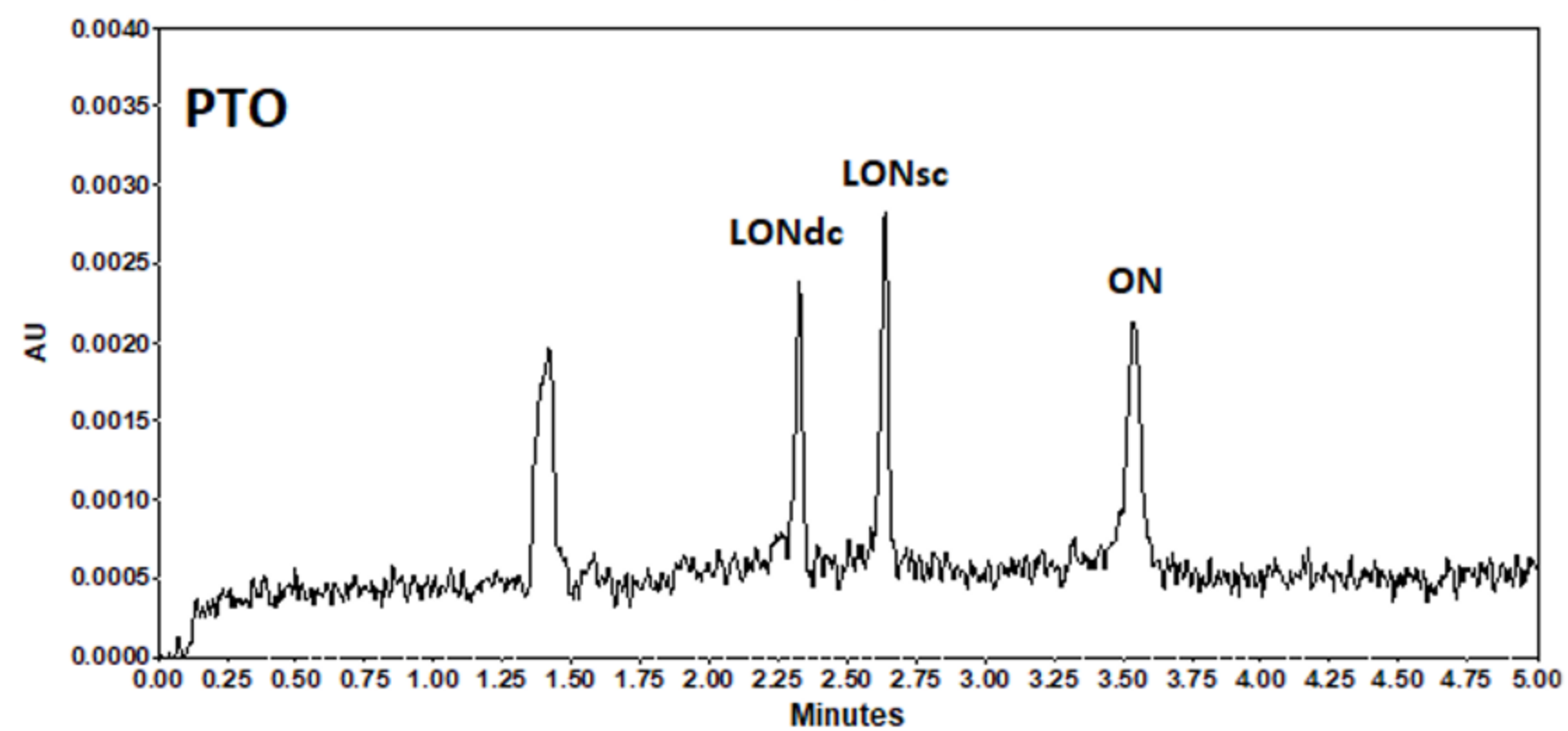
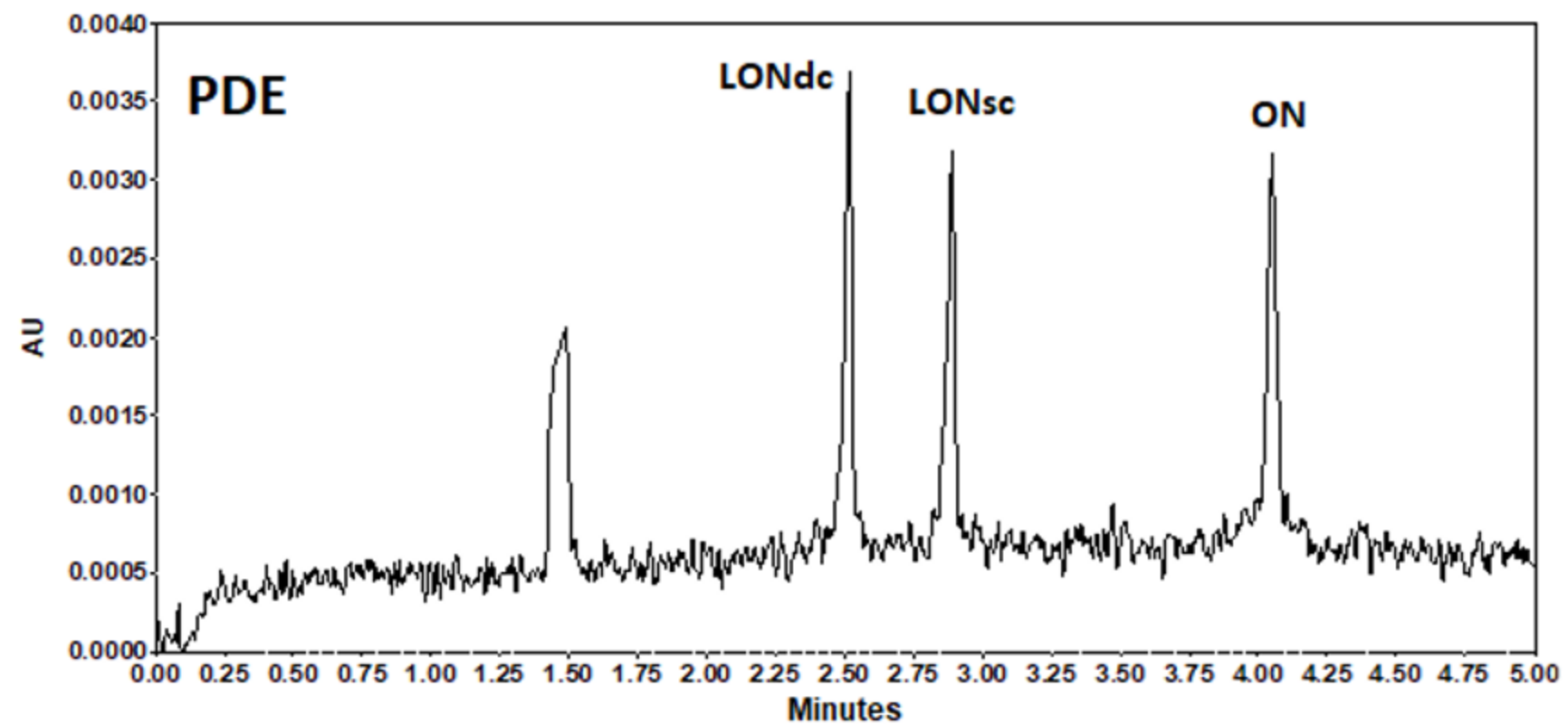
Figure 1: bar chart representing μ_{ep} absolute values for ON, LONsc and LONdc PDE and PTO, depending on the type of CD. Bare fused-silica capillary, 50 μm I.D. x 40.2 cm (UV detection at 30.2 cm). BGE: 10 mM sodium tetraborate buffer (pH = 9.5) with 15 mM CD. Applied voltage: +20kV; capillary temperature: 25°C; hydrodynamic injection: 0.5 psi, 10 s. UV detection at 254 nm. ONs and LONs at 10 μM in water. Analyses performed in triplicate ($n = 3$).

Figure 2: electrophoretic separation of ON, LONsc, and LONdc PDE and PTO. Bare fused-silica capillary, 50 μm I.D. x 40.2 cm (UV detection at 30.2 cm). BGE: 10 mM sodium tetraborate buffer (pH = 9.5) with 10 mM Me- β -CD. Applied voltage: +20kV; capillary temperature: 45°C; hydrodynamic injection: 0.5 psi, 10 s. UV detection at 254 nm. ONs and LONs in mixture at 5 μM each in water.

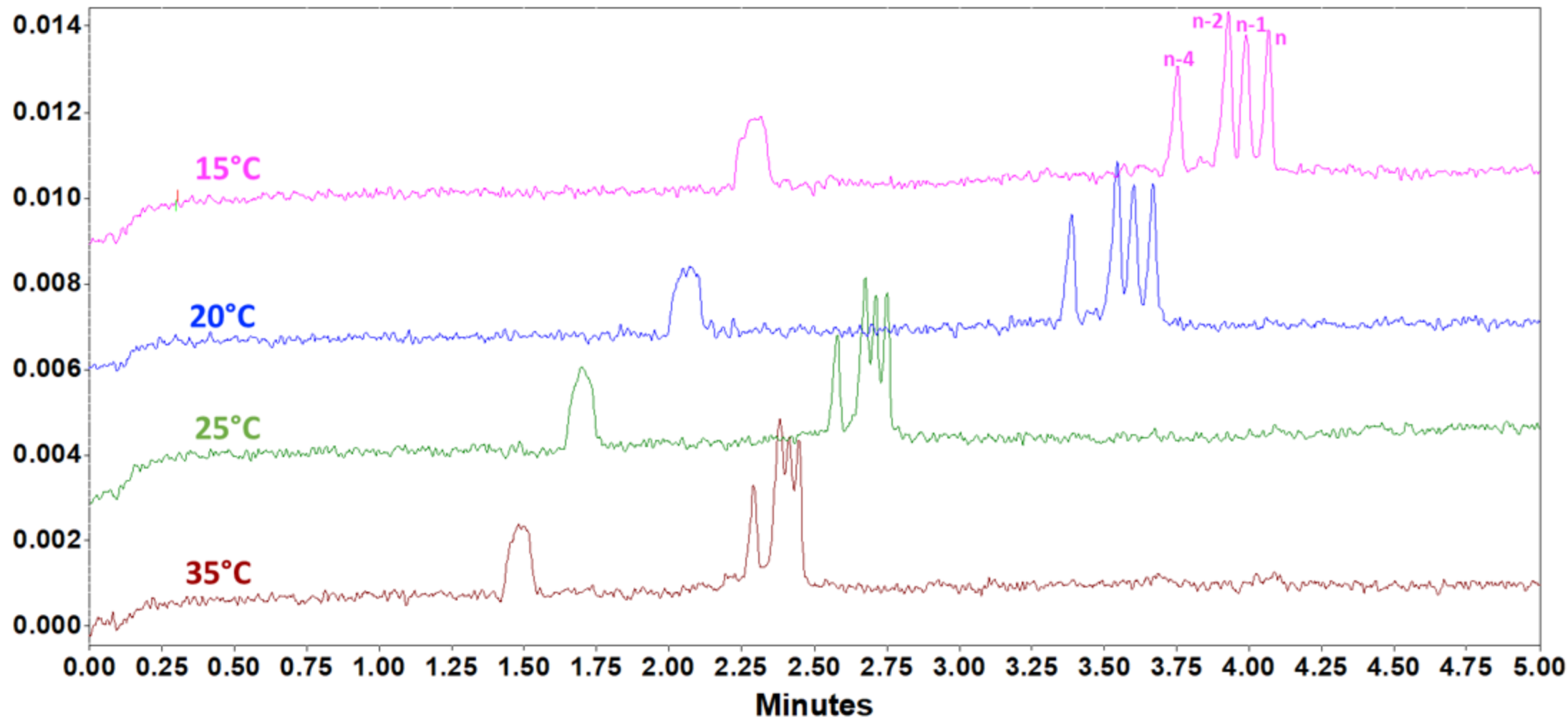
Figure 3: electropherograms of the separation between full-length sequence (n), (n-1), (n-2), and (n-4)-mers for LONdc PDE. Bare fused-silica capillary, 50 μm I.D. x 40.2 cm (UV detection at 30.2 cm). BGE: 10 mM sodium tetraborate buffer (pH = 9.5) with 10 mM Me- β -CD. Applied voltage: +20kV; capillary temperature between 15°C and 35°C; hydrodynamic injection: 0.5 psi, 10 s. UV detection at 254 nm. LONs in mixture at 10 μM each in water.

Figure 4: non-linear regressions (Eq.2) of the binding isotherms for LONsc and LONdc PDE and PTO. Bare fused-silica capillary, 50 μm I.D. x 40.2 cm (UV detection at 30.2 cm). BGE: 10 mM sodium tetraborate buffer (pH = 9.5) containing 0-0.6 mM Me- β -CD. Applied voltage: +20kV; capillary temperature: 25°C; hydrodynamic injection: 0.5 psi, 10 s. UV detection at 254 nm. LONs at 10 μM in water.





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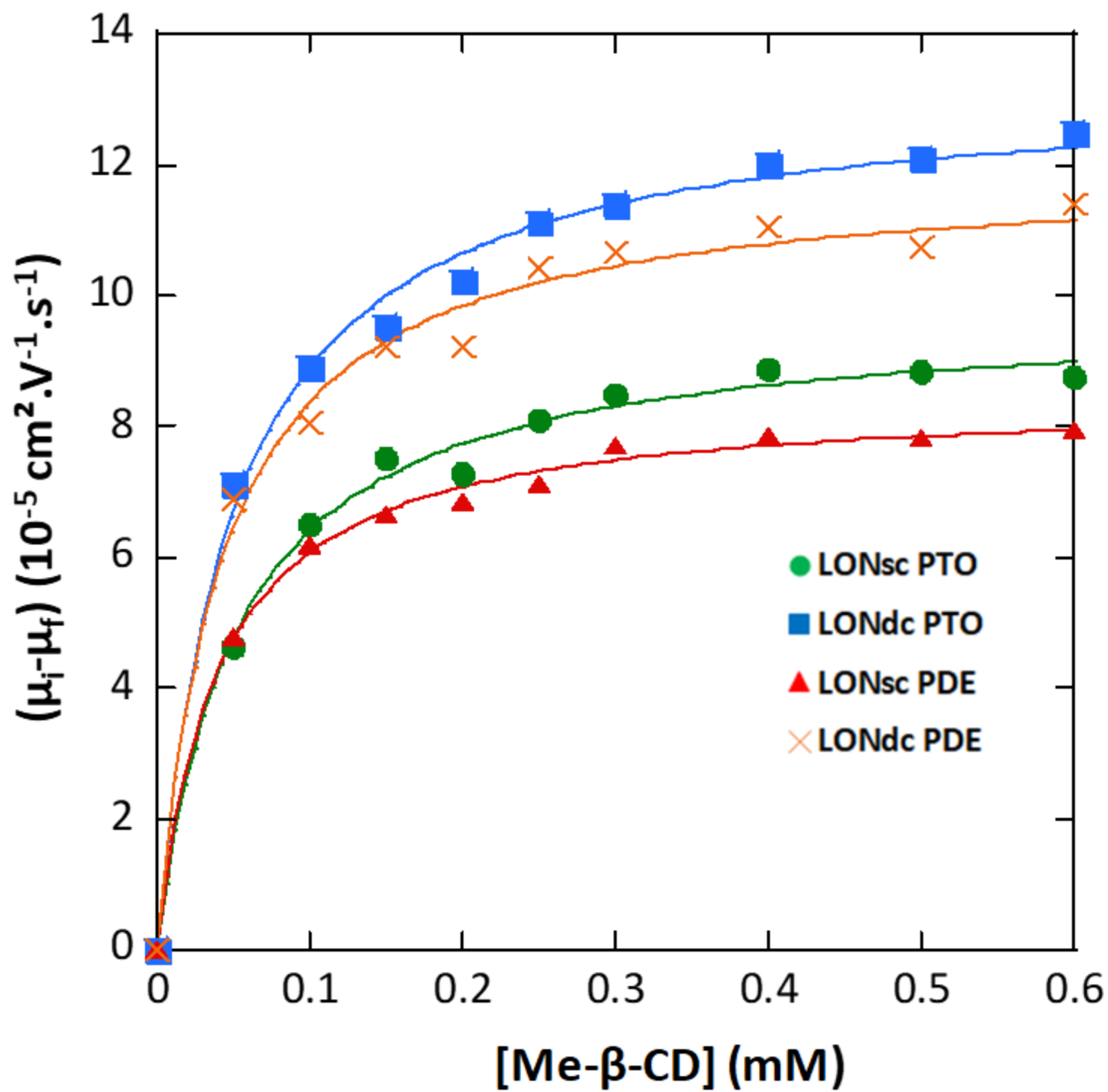
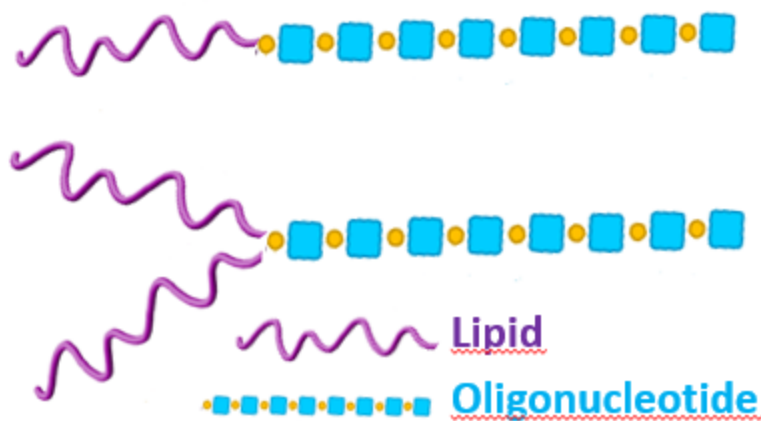


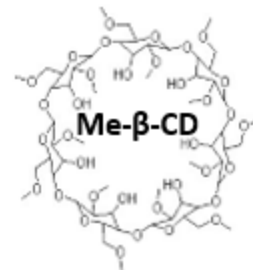
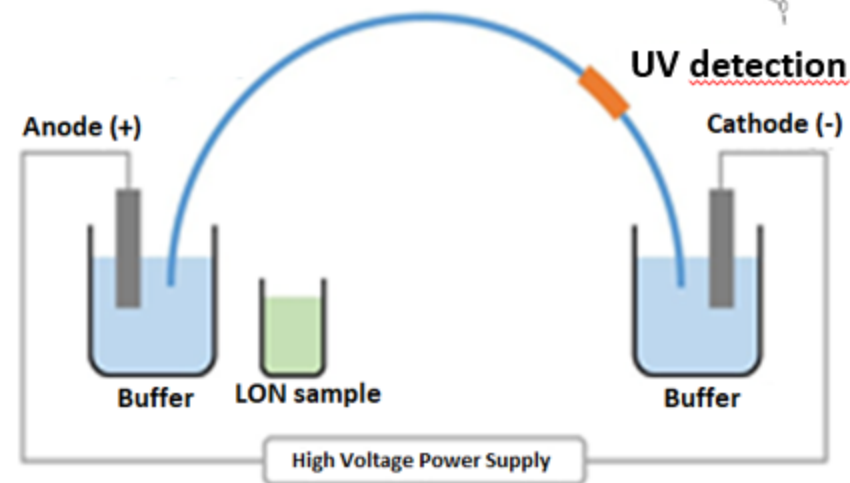
Table 1. Data for method performance assessment

| Analytes | Intra-day (n = 3) | | Inter-day (n = 3) | | Linearity | | LOQ μM | LOD μM | Accuracy |
|-----------|-------------------|------|-------------------|------|----------------|---------------|-----------|-----------|------------|
| | Area | | Area | | R ² | Range (μM) | | | Recovery % |
| | Mean (AU.min) | %RSD | Mean (AU.min) | %RSD | | | | | |
| ON PDE | 2534 | 1.28 | 2574 | 1.37 | 0.9985 | 5.1-30.1 | 2.9 | 1.0 | 95.3 |
| LONsc PDE | 2529 | 2.00 | 2791 | 2.48 | 0.9986 | 4.8-28.8 | 1.7 | 0.6 | 99.2 |
| LONdc PDE | 3657 | 1.24 | 3629 | 2.08 | 0.9982 | 5.8-35.0 | 2.9 | 1.0 | 96.5 |
| ON PTO | 1783 | 0.66 | 1725 | 2.06 | 0.9850 | 5.4-32.2 | 8.9 | 3.0 | 106.6 |
| LONsc PTO | 1801 | 1.73 | 1773 | 2.48 | 0.9981 | 5.4-32.3 | 5.0 | 1.7 | 99.9 |
| LONdc PTO | 1855 | 1.20 | 1870 | 6.13 | 0.9923 | 4.8-29.0 | 4.0 | 1.3 | 89.6 |

Lipid-Oligonucleotide conjugates (LONs)



Capillary Electrophoresis Analysis



LON separation LON-CD binding constant and complex mobility LON quantitative analysis

