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Study of nucleic acid–ligand interactions by capillary electrophoretic techniques: A review

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ABSTRACT

The understanding of nucleic acids–ligand (proteins, nucleic acids or various xenobiotics) interactions is of fundamental value, representing the basis of complex mechanisms that govern life. The development of improved therapeutic strategies, as well as the much expected breakthroughs in case of currently untreatable diseases often relies on the elucidation of such biomolecular interactions.

Capillary electrophoresis (CE) is becoming an indispensable analytical tool in this field of study due to its high versatility, ease of method development, high separation efficiency, but most importantly due to its low sample and buffer volume requirements. Most often the availability of the compounds of interest is severely limited either by the complexity of the purification procedures or by the cost of their synthesis.

Several reviews covering the investigation of protein–protein and protein–xenobiotics interactions by CE have been published in the recent literature; however none of them promotes the use of these techniques in the study of nucleic acid interactions. Therefore, various CE techniques applicable for such interaction studies are discussed in detail in the present review. The paper points out the particular features of these techniques with respect the estimation of the binding parameters, in analytical signal acquisition and data processing, as well as their current shortcomings and limitations.

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1. Introduction

Whether it gets to the interactions between the elements of the biosphere or down to the interactions within cells, bio-interactions are one of the driving forces of life. Some of the most common types of such interactions include: antibody–antigen reactions, receptor activation by an agonist, DNA translation or the activation or inactivation of different genes.

Nucleic acids are large biomolecules contained in the chromosomes of the living organisms and viruses. Nucleotides are the building blocks of nucleic acids, each one having three components: a pentose, a phosphate group and a nitrogenous base. Deoxyribose, the pentoze in DNA is replaced by ribose in RNA. The nucleic acids have different purposes within the cells and viruses, from encoding and storing the genetic or other type of information with very high efficiency (1 g of DNA can code up to 2.2 petabytes of data [1]), to transmitting and expressing this encoded information. Unfortunately, along with genetic information, a number of diseases can be also transmitted [2].

A mutation within nucleic acids, usually within DNA, is related to a change in the nucleotide sequence, caused either by base deletion, addition or base change. If natural DNA repair mechanisms are not activated, the damage will propagate further, potentially leading to abnormalities in the encoded protein [3]. When a specific protein indispensable for the functionality of the organism is affected, the symptoms of a disease may appear [4].

Nowadays, there are a number of diseases, ranking from cancer to viral infections, that are treated with drugs that act on the nucleic acids as target. [5]. These drugs include, but are not limited to: intercalating agents (doxorubicin, dactinomycin), alkylating agents (cisplatin, dacarbazin), chain cutters (calicheamicin), chain terminators antivirals (acyclovir, deoxyguanosine) and antisense oligonucleotides [5].

The screening stage in the development process of a new drug consists in the evaluation of the interaction of different synthetized molecules with the target receptor. The quantitative aspect of this interaction is usually described by the affinity constant and reaction stoichiometry.

The affinity constant, K_a is an equilibrium constant describing a system where an association–dissociation reaction takes place between and receptor (R) and a ligand (L) with the formation of a complex (C). The use of *ligand* and *receptor* is arbitrarily associated, without being a clear definition for each.

The general reaction can be summarized as follows:

$$R+L \rightleftharpoons C \tag{1}$$

The reaction is also characterized by the on-rate constant $k_{on}$and off-rate constant, k_{off}:

$$R+L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C \tag{2}$$

This can be converted to the following differential equation:

$$\frac{d[C]}{dt} = [R] \times [L] \times k_{on} - [C] \times k_{off} \tag{3}$$

At equilibrium, $\frac{d[C]}{dt} = 0$, so $[R] \times [L] \times k_{on} = [C] \times k_{off}$, thus by arranging the equation, K_b can be expressed as:

$$K_b = \frac{k_{on}}{k_{off}} = \frac{[C]}{[R] \times [L]} \tag{4}$$

The two reaction constants, the on-rate constant k_{on} and the off-rate constant k_{off}, have units of 1/(concentration × time) and 1/time, respectively.

The analytical techniques currently used for the study of biomolecular interactions in general and nucleic acids interactions in particular can be divided in two separate groups, namely mixture based and separation based techniques. In mixture based techniques, the affinity constants can be estimated by means of UV and Fourier transform infrared spectroscopy (FTIR) [6,7], nuclear magnetic resonance (NMR) [8], mass spectrometry [9,10], Raman spectroscopy [11–13], spectrofluorimetry [14], equilibrium (competition) dialysis [15–18], surface plasmon resonance [19,20] and ultracentrifugation [21]. The separation based techniques include techniques such as liquid chromatography (LC) and electrophoresis.

The use of liquid chromatography (i.e. HPLC techniques) exploiting biomolecular interactions was firstl described in the late 60′s for the separation and purification of enzymes and antibodies [22,23]. Currently, there are several chromatographic techniques available for the biomolecular interaction studies, such as frontal affinity chromatography, zonal affinity chromatography or Hummel–Dreyer analysis [24,25]. However, except for the case of preparative purposes, the volumes of sample (μL range) and mobile phase (hundreds of mL) required by the chromatographic techniques are most often too high. This can be even more critical in the case of minute amounts of pure ligand and/or receptor whose availability is limited, either due to tedious purification or synthesis procedures or to elevated costs of purchase. Therefore,, due to its inherently low sample (nL range) and buffer (few mL) volume requirements, CE represents a more appropriate alternative for the study of biomolecular interactions.

As compared to LC, CE is a relatively new separation technique, and, besides the low sample and running buffer consumption, it offers other numerous advantages that include high separation efficiency and ease of method development. All these features recommend its use for the study of nucleic acid–ligand interaction where one of the components is scarce or not available in a pure state.

So far, several reviews have dealt with the application of CE in the study of biomolecular interactions, yet none of them focused on the particularities of CE in nucleic acid studies.

Among these reviews, Busch et al. [26] were the first to compare different capillary electrophoresis techniques for the study of interaction between protein and ligand (warfarin and human serum albumin) pointing out the difference between them, as well as emphasizing that using different techniques might have an influence on the value of calculated affinity constant. Rundlett et al. [27] published another noticeable review describing several techniques (affinity capillary electrophoresis, Hummel–Dreyer, frontal and vacancy peak analysis) along with their advantages, limitations and practical applications.

Other publications are less focused, dealing in general with the topic of CE in the study of biomolecular interactions [28–39], however none of them covered its application in the study of nucleic acid–ligand interactions.

Table 1
Different CE techniques used for biomolecular studies.

Technique	Sample plug	Running buffer	Data of interest	Remarks	Ref.
CZE	Analyte + ligand	Running buffer	Peak area or peak height	– Usually suitable for slow on–off kinetics and strong interactions	[54]
ACE	Analyte	Ligand + running buffer	Migration time	– Suitable for fast on–off kinetics and weak and strong interactions – Ligand consuming	[52,53,55]
HD	Analyte and analyte + ligand	ligand + running buffer	Peak area or peak height	– Methodology similar to ACE – Suitable for fast on–off kinetics – Ligand consuming	[56,57]
FA	Analyte + ligand	Running buffer	Plateau height	– Methodology similar to CZE – Suitable for fast on–off kinetics	[58]
VP	Running buffer	Running buffer + analyte + ligand	Peak area or peak height	– Analyte and ligand consuming – Suitable for fast on–off kinetics	[56]
VACE	Running buffer	Running buffer + analyte + ligand	Migration time	– Methodology similar to VP – Analyte and ligand consuming – Suitable for fast on–off kinetics	[59–61]

The goal of the present review is to fill this gap, focusing on the use of capillary electrophoresis as a convenient analytical tool in determining the interactions of nucleic acids with different types of ligands. The principles and particularities of CE techniques employed for biomolecular interactions studies will be presented along with their reported applications and types of ligand-nucleic acid pairs tested.

2. Methodology

2.1. Capillary type

Due to its relatively low costs and easy maintenance, bare fused silica is the most common capillary used in CE. However, the charged inner wall exhibits some major disadvantages. Due to the negatively charged silanol moieties, large analytes tend to adsorb during separation, reducing the recovery and performance of the capillary [40]. In addition, bare fused silica capillaries develop an electroosmotic flow (EOF) which begins to be noticeable at a pH higher than 3, and going through an exponential growth at higher values. Moreover, the EOF shows a hysteresis effect, making it hard to control and posing reproducibility problems for certain applications [41,42].

Due to its simplicity, dynamic coating is an alluring method to prevent adsorption phenomena and to control the EOF. It is usually performed by pre-rinsing the capillary with a positively charged compound or with a polymer. They adsorb to the negatively charged silanol groups, suppressing the EOF and the adsorption of the analytes. Because of the physical nature of the surface modification mechanism, a small concentration of coating agent is added to the background electrolyte to keep the coating intact during separation [43]. This is necessary especially for low mass coating agents, whereas polymeric agents tend to keep the layer intact even after several runs [44,45]. However, the necessity to add the coating agent to the running buffer could be a hindrance when testing analytes that could also interact with it.

The permanent wall coating presents a more attractive method to eliminate the EOF and the adsorption of the analyte to the inner wall of the capillary. Contrary to the dynamic coating, it does not require regeneration or the addition of any reagent to the running buffer. The preparation of a permanent coating usually requires a several steps: a capillary pretreatment to activate the inner wall, introduction of double bonds to the inner wall (usually by a silane derivative) and polymerization of the double bonds with a monomer and a crosslinker [46].

Although covalently bonded capillaries exhibit numerous advantages, there are only a few types of coatings currently commercially available: linear polyacrylamide (LPA) [47], polyvinyl alcohol (PVA) and fluorocarbon (FC) [48,49].

2.2. Internal standard

The injection step, either hydrodynamic or electrokinetic, is a critical aspect in capillary electrophoresis. The use of an internal standard is able to correct variations in the injected sample volume, as well as it can significantly enhance the precision in assessing the target compound's migration time, when an accurate determination of the electrophoretic mobility is necessary (i.e. determination of pKa by CE) [50]. Internal standards (e.g. mesityl oxide, horse heart myoglobin) were employed in several ACE experiments [51–53], both to compensate for EOF variations and to estimate the value of effective mobilities.

Nevertheless, caution should be taken because, apart from their beneficial effect, internal standards employed in affinity capillary electrophoretic techniques may interact with one of the components of the studied system (especially with the biomacromolecule-protein, nucleic acid, etc.), affecting the accuracy of calculated binding parameters, even though no concerns related to this hypothesis has been reported so far.

2.3. CE techniques for bioaffinity studies

There are currently six types of CE techniques sharing various levels of similarity, that can be used for the determination of binding parameters in bioaffinity studies (i.e. drug–protein, protein–protein, protein–nucleic acid, etc.): capillary zone electrophoresis (CZE), affinity capillary electrophoresis (ACE), frontal analysis techniques (FA), vacancy peak technique (VP), vacancy affinity electrophoresis (VACE) and Hummel–Dreyer technique (HD) [26,27,30]. These techniques can be differentiated on the basis of the binding parameter that can be extracted from the raw data (Table 1). Thus, dependent upon the employed technique, the binding information can be extracted from the peak area (VP, HD, CZE), the height of the peak or plateau (FA) and from the change of the migration time (ACE, VACE). Since all commercially available CE instruments are equipped by default with a UV/VIS detector, thereby most frequently direct UV detection is reported in the bioaffinity studies involving CE techniques. Details regarding these techniques will be presented hereafter, with their key points being summarized in Table 1. Fig. 1 gives a schematic overview of the principles of CE techniques useful in biomolecular interaction analysis.

Most of the CE techniques described in this paper (affinity chromatography, Hummel–Dreyer [24], frontal analysis [62,63]

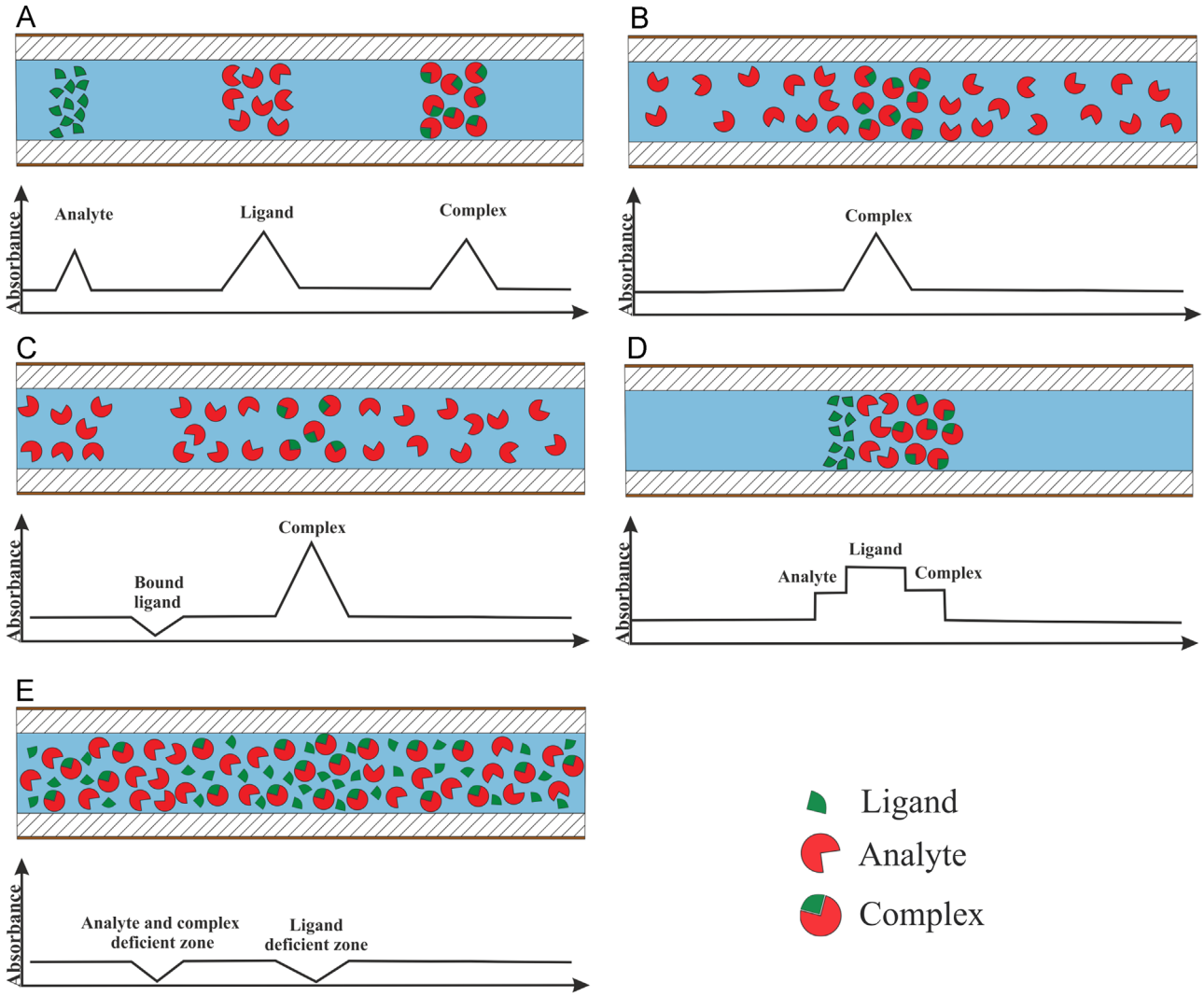

Fig. 1. Schematic principle of CE techniques employed in biomolecular interaction analysis (A. Capillary zone electrophoresis, B. Affinity capillary electrophoresis, C. Hummel–Dryer technique, D. Frontal analysis technique, E. Vacancy peak technique and Vacancy affinity capillary electrophoresis).

and vacancy peak [64]) were methods developed for gravity-feed size exclusion chromatography, subsequently being transferred to HPLC and later adapted to CE.

Affinity chromatography implies the use of a stationary phase functionalized with a ligand, which can be a protein, a sequence of nucleic acid (RNA or DNA) or a small molecule. Affinity chromatography is currently used either for separation purposes [65] or kinetic studies [66].

Compared to its electrophoretic counterpart, affinity chromatography shows a number of advantages and disadvantages. One of the main advantages, consist in the overall high accuracy, precision and reliability of the HPLC instrumentation, in terms of sample volume handling and flow control of the mobile phase. Another benefit, nevertheless coming at much higher costs, may be accounted in a long-term reproducibility of the recorded chromatographic behavior, where the ligand is being chemically bonded to the stationary phase. On the other hand, it is less versatile and cost-effective compared to ACE, where numerous ligands can be subsequently tested on the same capillary.

2.3.1. *Capillary zone electrophoresis (CZE)*

In CZE (Fig. 1A) the resulting complex must have different electrophoretic mobility (μ) as compared to the mobility of the ligand and the analyte (i.e. nucleic acid). This technique can be used in studies of strong interactions, where the complex is stable enough for the duration of the analysis. Depending on the nature of the ligand and of the analyte, as well as on their molar ratio, up to three peaks can be observed: one for the ligand, one for the analyte and one for the forming complex. Usually a pre-incubation phase is required, in which a known concentration of analyte is incubated with increasing concentrations of ligand. By using an independent calibration curve, the concentration of free and bound ligand can be determined. These values can be used in conjunction with the classical Scatchard method to determine the binding constant, which is the negative slope of the plot representing the ratio of bound ligand to unbound ligand concentration as a function of bound ligand concentration.

2.3.2. *Affinity capillary electrophoresis (ACE)*

Chu et al. were one of the first to use ACE for the determination of affinity constants of carbonic anhydrase B with 4-alkyl benzensulfonamides derivatives [55]. This method was quickly adopted by others in affinity studies experiments [52,53,55].

In order to employ the ACE technique (Fig. 1B), the analyte and the complex must exhibit a different mobility and the equilibration time must be lower than the separation time. This technique can be used for the study of systems with weak interactions but fast kinetics. Increasing concentrations of ligand are added to the buffer and the mobility of the analyte is monitored.

The mobility of the analyte will shift between two values. At the beginning the analyte will have a maximum value of mobility, corresponding to the analyte in the absence of the ligand in the sample $\mu_{A,0}$. As the ligand's concentration in the buffer increases, the analyte mobility will decrease down to a minimum, $\mu_{A,\ Lmax}$. The affinity constant can be extracted either by using a variation of the Scatchard method (plotting the $\Delta\mu$/[ligand] as a function of $\Delta\mu$, where $\Delta\mu$ is the change in electrophoretic mobility, the affinity constant being estimated as the negative slope) or by fitting the data by a non-linear regression ($\Delta\mu$ as a function of [ligand]).

Using ACE, higher concentration (at least one order of magnitude) of ligand in the running buffer should be employed in comparison with that of the analyte from the injected sample [67].

2.3.3. Hummel–Dreyer (HD) technique

The HD technique (Fig. 1C) was, as explained before, first introduced in liquid chromatography [24], being later transferred for use in capillary electrophoresis.

This technique is particularly suitable for systems with weak interactions, and it implies the use of a buffer containing the ligand at a known concentration. The sample consists of the analyte and the ligand at a lower concentration than that in the buffer.

The amount of bound ligand can be calculated either using the internal or the external calibration method. In the case of the internal calibration method, the ligand's concentration in the sample is steadily increased to a concentration equal to and eventually higher than its concentration in the buffer. At zero or low concentration of ligand in the sample, the electropherogram exhibits a positive peak, corresponding to the complex, and a negative peak corresponding to the difference in concentration of the ligand in the sample and in the buffer. By increasing the concentration of ligand in the sample, the negative peak will decrease and eventually become positive. By interpolation, the concentration of ligand at which the corresponding peak disappears can be estimated, which in turn allows for the assessment of the bound amount of ligand. The external calibration method uses the area of the negative peak upon injecting a blank buffer and the area of the negative peak upon injecting the analyte. The amount of bound ligand can be estimated from the difference in these two areas.

In the HD technique, the concentrations of the analyte and of the ligand are both known, which implies that the stoichiometry can also be calculated. The affinity constant can be calculated from the Scatchard plot of [bound ligand]/[free ligand] as a function of [bound ligand].

2.3.4. Frontal analysis (FA) technique

This method (Fig. 1D) can be used when the analyte and the complex have similar electrophoretic mobility, but different from that of the ligand. The capillary is filled with blank buffer and a large plug of sample containing the analyte and the ligand is injected. The electropherogram consists of two or three plateaus depending on the mobility of the analyte, the complex and the ligand, respectively. The height of each plateau reflects the concentration of its component. The free ligand concentration can be calculated by an external calibration method. By knowing the initial concentration and the reminder (free) concentration of ligand, the affinity constant can be estimated by Scatchard analysis as mentioned above.

2.3.5. Vacancy peak (VP) technique

The VP techniques (Fig. 1E) can be used when the electrophoretic mobility of the analyte and the complex are different from that of the ligand.

In this technique, the capillary is filled with the running buffer containing a mixture of analyte, ligand and their complex in a dynamic equilibrium. By injecting a plug of blank buffer, two negative peaks appear induced by the differences from the background signal. One is corresponding to a ligand deficient zone (with its area dependent on the concentration of bound ligand in the buffer), whereas the second peak corresponds to an analyte and complex deficient zone (with its area dependent on the free ligand's concentration).

The concentration of the free ligand can be estimated by an internal calibration method, injecting samples of buffer with increasing concentrations of ligand and fixed amount of analyte. As the concentration of ligand in the sample increases, the negative peak corresponding to the free ligand decreases and eventually becomes positive. By data interpolation, the concentration of ligand at which there is no peak can be calculated, which indicates the concentration of ligand bound to the analyte for a stoichiometric interaction. Thus, both the stoichiometry and the affinity constant can be estimated by this technique.

2.3.6. Vacancy affinity capillary electrophoresis (VACE)

The methodology for VACE is similar to the one discussed in VP (Fig. 1E), but the method of extracting the information from the raw data (migration times) corresponds to the one discussed at ACE [59]. As in the case of VP, the capillary is filled with a buffer containing a mixture of analyte and ligand in a dynamic equilibrium. Once again, upon injecting a small plug of blank buffer, two negative peaks will arise as discussed at the VP technique.

The affinity constant is extracted either by using the variation of the Scatchard method or by fitting the data by a non-linear regression.

3. Types of interactions involving nucleic acids analyzed by CE

Due to their secondary, tertiary and quaternary structures [3], the nucleic acids are able to exhibit specific binding sites for various molecules. Through the binding at these sites, different molecules (i.e. ions, steroids, proteins) can modulate the activity of the nucleic acid.

These types of interactions are important because they are involved in cell growth and cellular communication, but also as part of the cytotoxic mechanisms of certain drugs.

3.1. a) Nucleic acid–protein interactions

The interactions between proteins and nucleic acids have an important role in the functionality of the cell. Some examples of nucleic acid–protein interactions analyzed by CE are presented in Table 2.

tRNA plays an important role in the conversion of the DNA information to proteins and can also interact with different proteins during the synthesis process. Malonga et al. [68] investigated the interaction between tRNA and human serum albumin using

Table 2
Nucleic acid–protein interactions analyzed by CE.

Analyte	Ligand	Technique	Capillary	Ref.
tRNA	Human serum albumin	ACE	Bare fused silica	[68]
ssDNA	E.coli, L. acidophilus	CZE, ACE	Bare fused silica	[69]
$tRNA^{Phe}$, ASL^{Phe}-Cm_{32}, Gm_{34}, m^5C_{40}	t^F2, Tat1, Tat5	CZE	LPA coated	[70]
$tRNA^{Phe}$	Phage peptides	CZE	LPA coated	[71]
ssDNA	kin17 protein	ACE	PEO dynamic coated fused silica capillary	[72]

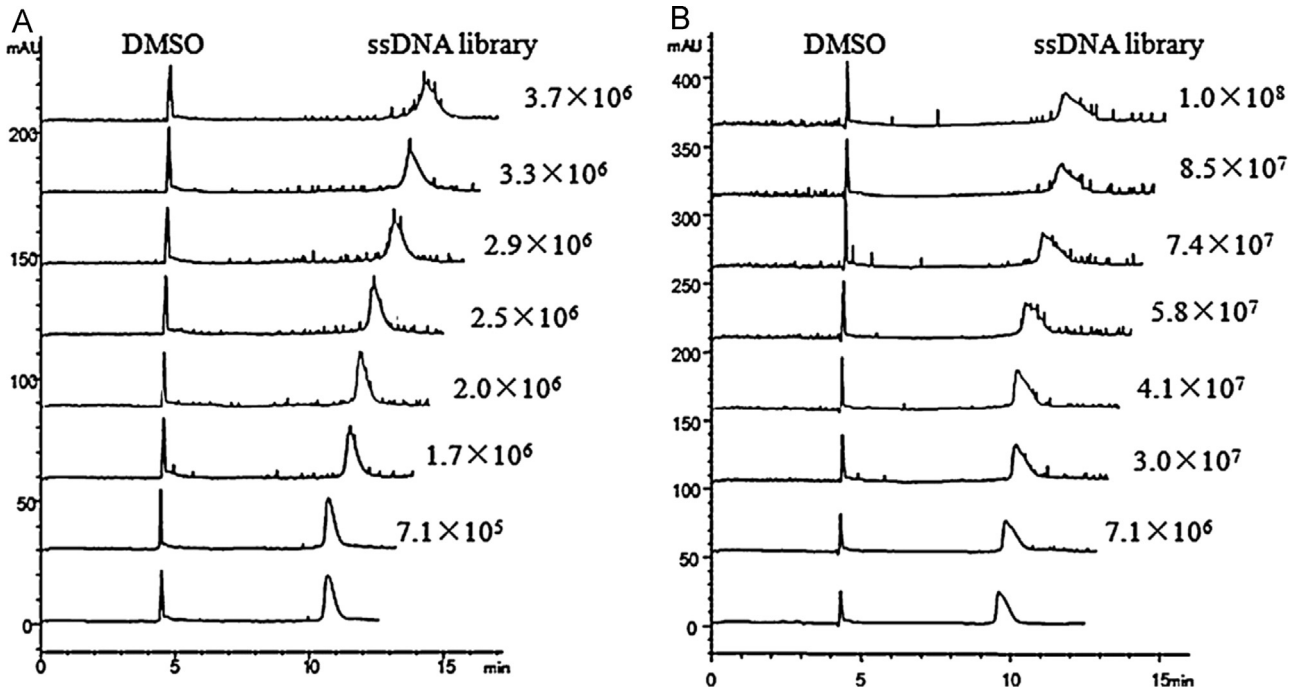

Fig. 2. Electropherograms of 10 μM ssDNA library (sample) in buffer with increasing concentrations of L. acidophilus protoplasts (A) and E. coli protoplasts (B). The concentration of protoplasts is in CFU/mL. Reprinted from Ref. [69], with permission.

ACE at physiological-like conditions (phosphate buffer 12.5 mM at pH 7.5). The RNA concentration was kept constant and the HSA was added to the buffer at different concentrations (between 0.04 and 0.6 M). The analyses were carried out using bare fused silica capillary and allow to calculate an estimated value for the affinity constant of $K_a = 1.45 \times 10^4\ M^{-1}$ showing a one phase interaction between the tRNA and HSA. As a result of the interaction, there is a slight increase in proportion of α-helix form which could indicate a structure stabilization.

For the screening of the most suitable high affinity biocomponent in the development of aptasensors for the selective detection of various bacteria, Meng et al. [69] reported the use of ACE and CZE for the study of interaction between ssDNA library and Escherichia coli or Lactobacillus acidophilus (Fig. 2). Protoplasts of these bacteria show greater affinity for the ssDNA compared to the corresponding bacteria and that by treating the bacteria with different solvents selective affinities are to be observed for different ssDNA strands. Bare fused silica capillary was employed, revealing similar affinity constants by ACE and CZE. Considering the fact that proteins can modulate nucleic acids, Mucha et al. [70] used CZE with a LPA coated capillary and a buffer with a soluble matrix (containing linear polyacrylamide) to investigate the interaction of tRNA with phage display peptides. The LPA coated capillary and the soluble matrix added to the buffer efficiently minimized the EOF and interaction with the capillary wall, offering high efficiency and well-shaped peak. Thus the method shows promising perspectives to be used for other RNA–protein interactions. In another set of experiments, the team studied the effect of of tRNA methylation on the interactions with proteins [71]. They used a similar setup as in the previous study and observed a positive binding effect of the methylation of RNA for some proteins and negative for others.

It is well known that the basic proteins have a strong affinity for the silica surface of the capillary. To test the interaction between the basic kin17 protein and ssDNA, Tran et al. [72] developed a dynamic coating procedure by using polyethylene glycol 200 k, thus suppressing both the EOF (to values as low as $5 \times 10^{-5}\ cm^2/V \times s$) and the adsorption of the protein to the charged wall surface. The procedure presented good RSD% for the migration time (0.3%) and a fair recovery (79%) of the aforementioned protein.

3.2. b) Nucleic acid-small ligand (small molecules) interactions

The purine and pyrimidine bases linked to the sugar-phosphate backbone of the nucleic acids may lead to coordination complexes with different transitional metal ions. Several examples of nucleic acid interactions with metal ions and other small molecules analyzed by CE are presented in Table 3.

Table 3
Nucleic acid-small ligand interactions analyzed by CE.

Analyte	Ligand	Technique	Capillary	Ref
Calf thymus DNA	Fe^{2+},Fe^{3+}	CZE	Bare fused silica	[73]
Calf thymus DNA	Polyamines, Cobalt(III) hexamine	CZE	Bare fused silica	[74]
Calf thymus DNA, Baker's yeast RNA	Ag(I)	CZE	Bare fused silica	[75]
HIV-1 Tat–TAR RNA	β-carboline, iso quinoline alkaloids	CZE	Bare fused silica	[76]
HIV-1 Tat–TAR RNA	β-carboline alkaloids	ACE	Bare fused silica	[77]
dsDNA	Netropsin	CZE, ACE	Bare fused silica LPA coated	[78]
dsDNA	Berberine	CZE	Bare fused silica LPA coated	[79]
DNA isolated from chicken erythrocytes	CdTe quantum dots	CZE	Bare fused silica	[80]

Iron ions are known to be involved in DNA oxidation, either directly or by peroxide mediated oxidation. Ouameur et al. [73] studied the interaction between calf thymus DNA and Fe^{2+} and Fe^{3+} ions. They employed CZE with a bare fused silica capillary and a running buffer containing 15 mM Tris–HCl pH 6.5 and 15 mM NaCl, obtaining two affinity constants for each ion. By FTIR analysis, they concluded that the smaller constant is due to Fe–PO_2 interaction and the higher one due to the Fe–N_7 (from the guanine moiety) interaction. The interaction of DNA with these metal ions, especially at high concentrations, is accompanied by changes in the nucleic acid's structure, i.e. Fe^{2+}– DNA interaction induces helix destabilization, while Fe^{3+} causes DNA condensation.

The interaction of small molecules with the nucleic acids are particularly important because they are involved in cell growth and cellular communication, but also as part of the cytotoxic mechanisms of certain drugs.

Biogenic polyamines are important in cell proliferation and differentiation due to their interaction with nucleic acids. Ouameur et al. [74] investigated the interaction of spermine, spermidine, putrescine and cobalt(III)hexamine with calf-thymus DNA by capillary electrophoresis, FTIR and circular dichroism spectroscopy. They used a bare fused silica capillary and a 20 mM Tris–HCl (pH=7) solution as running buffer. The sample, containing a constant concentration of DNA and increasing concentration of polyamines was incubated at 25 °C before injecting. The FTIR data showed that at low concentrations, putrescine demonstrates affinity for both the minor and major groove of the DNA double strand, while spermine, spermidine and cobalt(III) hexamine bind only to the minor groove. On the other hand, at high concentrations, the putrescine's affinity is decreasing. Spectral analysis (FTIR and circular dichroism) indicated that spermine and cobalt(III) hexamine bind preferentially to the major groove while spermidine binds to both minor and major groove. Only cobalt(III)hexamine was able to change the DNA structure, inducing a partial transition in its geometry from form B to form A. The estimated affinity constants were: $K_{spermine}=2.3\times10^5\ M^{-1}$, $K_{spermidine}=1.4\times10^5\ M^{-1}$ and $K_{putrescine}=1.4\times10^5\ M^{-1}$, respectively. In case of cobalt(III)hexamine, two affinity constants were calculated: $K_1=1.8\times10^5\ M^{-1}$ and $K_2=9.4\times10^4\ M^{-1}$, respectively. The authors conclude that the beneficial stabilization of chromatin and thermal-, chemical- and radiation protective effect of polyamines over dsDNA is related to the high affinity interaction with these polycations.

The Ag^+ ion has strong antibacterial properties, being also involved in the interaction with the nucleic acids; however the reaction mechanism is not yet fully understood. Arakawa et al. [75] investigated the interaction of the Ag^+ with calf thymus DNA (mixture of double and single stranded) and baker yeasts RNA. They used bare fused silica capillary and 20 mM $NaClO_4$ as running buffer at 25 °C. The injected mixtures contained increasing concentrations of silver ion and a constant concentration of one of the two nucleic acids and were monitored by UV absorbance at 260 nm. In the case of the DNA, the Scatchard plot presents two distinctive slopes, suggesting two different binding sites with different affinity constants $K_1=8.3\times10^4\ M^{-1}$ and $K_2=1.5\times10^4\ M^{-1}$, while for the Ag^+-RNA complex there is only one slope corresponding to a value of $K=1.5\times10^5\ M^{-1}$. The FTIR data demonstrated that at low concentrations the silver ion binds to the N7 guanine of the DNA, however at high concentrations its binding site switches to N7 adenine. In the case of RNA the interaction with the silver ions is at the guanine base.

Ding et al. [76,77] evaluated the interaction between the transactivation element of the HIV virus type 1 mRNA and different natural alkaloids analogues (C_3, MC3, IG3, iso-C3). The RNA and the drugs were mixed in different proportions and incubated at 37 °C for an hour before injection. The separation conditions implied the use of a background electrolyte containing 50 mM phosphate buffer at pH=8 and a bare fused silica capillary (total length 57 cm, i.d.75 μm). The experiments were carried out at a constant voltage of +25 kV and temperature of 20 °C, the results showing that C_3 analogue having the highest affinity for the mRNA, followed by IG3 and MC3 having similar affinity with the iso-C3 analogue ($K_{C3}=50.4\times10^3\ M^{-1}$, $K_{MC3}=3.7\times10^3\ M^{-1}$, $K_{IG3}=8.56\times10^3\ M^{-1}$ and $K_{iso\text{-}C3}=4.33\times10^3\ M^{-1}$). The authors hypothesized that the aforementioned molecules have anti-HIV activity by inhibiting the formation of the complex between HIV-1 Tat and TAR RNA.

Netropsin is a well-known minor groove DNA binder with antiviral and antibiotic properties and therefore studying its interaction with the DNA is of interest. He et al. [78] investigated the affinity of netropsin for a 14mer dsDNA by means of CZE and ACE. They used a LPA coated capillary and highlighted the importance of the coating in attaining the desired performance of the method. In both CZE and ACE modes, a buffer containing 20 mM Tris-AcOH, 10 mM NaCl at pH 7.2 was used (Fig. 3). The estimated values for the affinity constant differ in the two techniques, for the CZE techniques a value of $1.07\times10^5\ M^{-1}$ was obtained, while for ACE a value of $0.47\times10^5\ M^{-1}$. A 1:1 M ratio of binding stoichiometry was estimated from the CZE analysis.

Berberine has antibacterial, anti-inflammatory and antineoplastic properties. Wu et al. [79] investigated the interaction of dsDNA with berberine using CZE with a coated and an uncoated capillary. Using a buffer composed of Tris-AcOH at pH 7.4, the determined binding constant of berberine with the dsDNA fragment was $K=1.0\times10^3\ M^{-1}$. They also reported the importance of capillary coating, where the LPA coated capillary assured a significantly higher performance in comparison with the bare fused silica.

Quantum dots offer interesting possibilities due to their special properties. Stanisavljevic et al. [80] investigated the interaction between dsDNA and CdTe quantum dots by using capillary electrophoresis with laser induced fluorescence detection. Due to their very small size (2 nm) the quantum dots can interact with the major groove of the dsDNA. The total absence of interaction with the ssDNA confirmed the reported mechanism of interaction.

3.3. c) Nucleic acid–nucleic acid interactions

The present literature does not cover classical CE studies

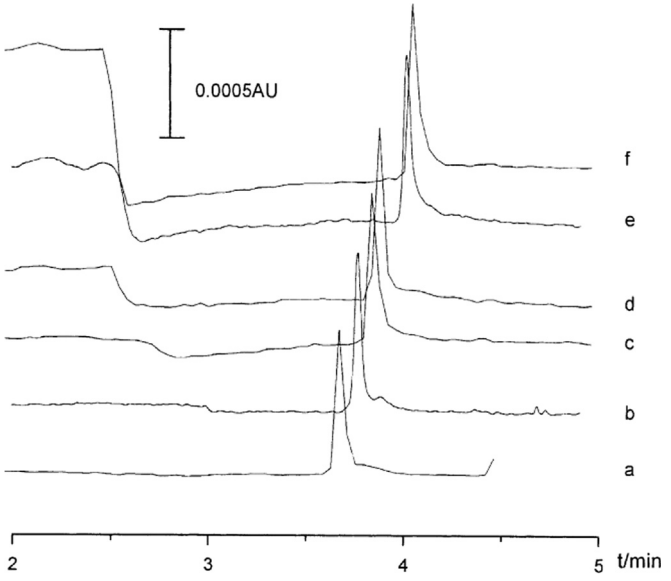

Fig. 3. Electropherograms of 1.4 mM 14mer dsDNA in buffer containing increasing concentration of netropsin: (a) blank buffer, (b) 5 μM netropsin, (c) 12 μM netropsin, (d) 20 μM netropsin, (e) 50 μM netropsin, (f) 75 μM netropsin. Reprinted from Ref. [78], with permission.

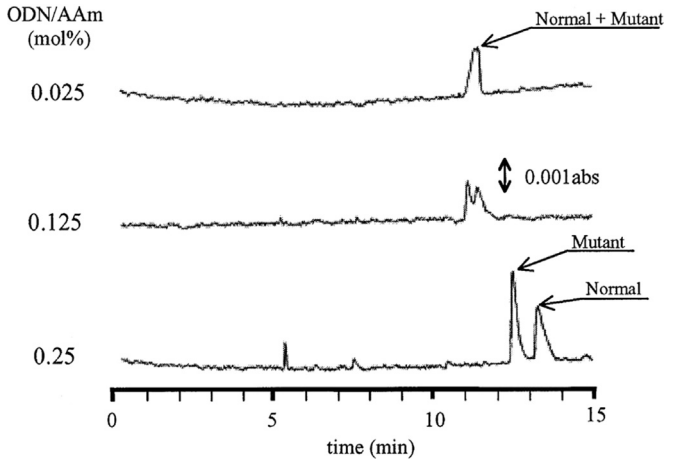

Fig. 4. The effect of affinity ligand concentration on the electrochromatographic separation of the normal and mutant sequences of DNA on nucleotide-functionalized open tubular capillary. Conditions: Capillary with an internal diameter of 75 μm, external diameter 375 μm, total length of 50 cm and effective length of 38 cm; buffer: Tris-borate 5 mM, 5 mM $MgCl_2$, pH=7.4.Injection of a sample (4.0 μM), 0.1 kgf/cm^2 for 1 s; electric field 300 V/cm, detection at 260 nm. Reprinted from Ref. [81], with permission.

focused on the interactions between nucleic acids. Nevertheless, Anada et al. [81] investigated the use of oligonucleotide-functionalized bare fused capillary for the separation of different DNA fragments. The ligand (antisense 6-mer DNA) was transformed into its methacryloyl derivative and it was copolymerized with 3-(methacryloyloxy)propyl trimethoxysilane moiety bound to the inner wall's surface in the presence of acrylamide as crosslinker. The nucleotide-functionalized open tubular capillary enabled the capillary electrochromatographic separation of the normal and mutant sequences of DNA in less than 15 min using a 5 mM Tris-borate buffer containing 5 mM $MgCl_2$ (pH=7.4) (Fig. 4). Similar to ACE, the concentration of ligand bound to the inner wall influence the migration time and thus the separation of the analytes.

4. Data analysis

The Scatchard analysis was one of the first used methods to extract data from binding systems and was later adapted for chromatographic and electrophoretic analysis. The equation this method relies on is:

$$\frac{r}{c}=nK_a-rK_a \tag{5}$$

where r is the ratio of bound ligand to total available binding sites, c is the molar concentration of ligand, n is the number of binding sites per molecule of analyte and K_a is the affinity constant of the reaction.

For a more straightforward application, the previous equation (5) can be transformed to:

$$K_a=\frac{[C]}{[A]\times[L]} \tag{6}$$

where [C] is the concentration of formed complex, [A] the concentration of free analyte and [L] the concentration of free ligand.

From the graphic representation of bound ligand divided by the concentration of free ligand as a function of bound ligand, the slope, $-K_a$, may be calculated. This method can be applied when the bound and free ligand concentrations are known, such as the CZE method.

For techniques where change in the electrophoretic mobility is registered, an adapted version of the Scatchard equation is used:

$$\frac{\Delta\mu}{[L]}=K_a\Delta\mu_{max}-\Delta\mu K_a \tag{7}$$

where $\Delta\mu$ is the difference between the electrophoretic mobility of the analyte recorded in the buffer void of ligand and the electrophoretic mobility of the analyte recorded in the presence of various concentrations of ligand, K_a is the apparent affinity constant and $\Delta\mu_{max}$ is the difference in mobility between the free analyte and analyte saturated with ligand. From the plot of $\Delta\mu/[L]$ as a function of $\Delta\mu$, the K_a can be extracted as the negative value of the slope.

Because data linearization during the Scatchard analysis may distort experimental errors leading to misinterpretations, more recently nonlinear regression became the preferred option.

For example, an equation used in ACE for the binding constant determination using nonlinear regression is:

$$\Delta\mu=K_a\times(\mu_{max}-\mu_0)\times([L]/(1+K_a\times[L])) \tag{8}$$

where $\Delta\mu$ is the difference of analyte mobility at a certain concentration of ligand in the buffer and its mobility in the absence of the ligand μ_0; K_a is the affinity constant, μ_{max} is the maximum theoretical difference in mobility and $[L]$ is the ligand concentration.

Currently data handling is performed using any commercially available, scientific data and graphing software packages, capable of nonlinear regression and data fitting, amongst which the most known are MatLab, Origin, GraphPad.

A recent mathematical approach enables the extraction of the binding constant and both the *on* and *off* kinetics constants [82]. This method is a derivative of the mathematical technique used in Macroscopic Approach for Studying Kinetics at Equilibrium (MASKE) and can be used in ACE were the ligand concentration is much higher than that of the analyte. The aforementioned technique implies the fitting of the whole electropherogram by nonlinear regression. This technique holds great promises due to the fact that it requires a reduced number of experiments and because beside the binding constant, it can determine both *on* and *off* kinetics constants.

5. Conclusions

The understanding of nucleic acids–ligand interactions is of great interest to the scientific community because they represent the basis of complex mechanisms that govern life and their full elucidation could lead to the development of better drugs and improved therapeutic strategies and might, as well, contribute to cure currently untreatable maladies. The techniques used to study these phenomena, can not only point out or confirm an interaction, but they can also be used to determine quantitative aspects like binding constant and stoichiometry.

Capillary electrophoresis has proven its usefulness in the field of separation techniques, but continues to be underused in biomolecular studies. As it has been shown, CZE and ACE are the only techniques currently reported in the literature, either because they share the longest history for such applications or because their simple experimental setup and data processing render them more accessible to less experienced users. Other available CE techniques discussed here might be better suited for particular cases of interactions involving nucleic acids, offering a more complex picture of the studied processes. Moreover, using multiple approaches might be able to point out the impact of potential experimental errors inherently affecting such type of determinations.

When selecting the appropriate electrophoretic technique for the determination of binding constant, different experimental variables (capillary type, coated vs. uncoated) and method particularities

(analyte/ligand requirements, fast or slow on/off kinetics, strong or weak interactions) should be taken into account. When starting these types of CE experiments, most often little is known about the type and rate of interaction between the nucleic acid and the ligand. Therefore, different types of CE approaches are advised to be performed for a better comprehension of the system's behavior and also to obtain the most accurate values of the studied parameters.

The data analysis usually requires the fitting of the experimental points by non-linear regression, however by this approach only the binding constant can be extracted and not the *on/off* kinetic constant. A more recent and promising approach for ACE, is the fitting of the whole electropherogram by nonlinear regression, which allows the calculation of both of these constants by requiring only a reduced number of experiments.

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