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Enantiomeric Sensing and Separation by Nucleic Acids

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Abstract

Naturally occurring polymers (and their derivatives) such as polysaccharides and proteins are very popular chiral selectors in analytical sciences. In contrast, nucleic acids have received much less attention in the enantiomeric analysis field. However, some significant advances have been accomplished during the last thirty years. The present review covers these different contributions in the development of both chiral sensor and separation systems. They rely on the use of either nonspecific nucleic acid molecules or target-specific oligonucleotides. The main practical factors as well as the applicability features of the reported nucleic acid-based enantioselective tools are summarized. Some possible routes for improvement are also suggested.

Keywords: chiral discrimination; nucleic acids; oligonucleotides; aptamers; enantiomers; sensors; liquid chromatography; capillary electrophoresis

List of abbreviations:

CE: capillary electrophoresis

CEC: capillary electrochromatography

EDTA: ethylenediaminetetraacetic acid

FAM: carboxyfluorescein

GC: gas chromatography

HEX: hexachlorofluorescein

HPLC: high-performance liquid chromatography

LC: liquid chromatography

MEKC: micellar electrokinetic chromatography

NMR: nuclear magnetic resonance

PVA: polyvinyl alcohol

SELEX: systematic evolution of ligands by exponential enrichment

SFC: supercritical fluid chromatography

TWJ: three-way junction

1. Introduction

The development of chiral assays for determining the enantiomeric purity of samples has found extremely wide applications in pharmaceutical, environmental or agrochemical sciences. Among the numerous analytical enantioselective methods reported to date [1], chromatographic (HPLC, SFC, gas chromatography, GC) as well as electro-driven (CE) approaches are undoubtedly the most popular ones as the result of their high accuracy, efficiency and robustness. Significant advances in terms of rapidity, simplicity and high-throughput capabilities have more recently come from designing chiral sensors mainly based on optical and electrochemical transduction techniques. Several excellent reviews discussing the last developments in these areas can be found elsewhere [2-5]. Both separation- or sensor-based enantioselective systems are typically based on chiral selectors of either synthetic or natural origin. The latter ones, which include either small molecular (amino acids, cyclodextrins, glycopeptides, alkaloids etc...) or macromolecular (polysaccharides, proteins) receptors, are especially important in analysis. For example, the polysaccharide derivatives are the most employed chiral selectors in the specific HPLC and SFC chiral separation [2].

On the other hand, the other essential biopolymers such as nucleic acids have received much less attention as chiral selectors and are not yet fully explored in enantiopurity analysis. Nonetheless, a variety of works in other scientific fields have revealed that the chirality of DNA and RNA molecules has impressive implications in asymmetric catalysis [6], medicinal chemistry and drug design [7, 8], nanotechnology [9] or structural biology [10]. Moreover, some reviews dealing with the impact of the nucleic acid chirality in (bio)chemistry have been previously published [11, 12]. However, to the best of our knowledge, the specific topic related to the nucleic acid molecule-based chiral analysis methodologies has not been yet reviewed.

In this paper, we aim at highlighting the advances accomplished in the field during the last thirty years. We will focus on the two main categories of chiral selectors that comprise (i) the nonspecific nucleic acid molecules without pre-defined analyte selectivity and (ii) the target-specific aptamer oligonucleotides specifically selected against the racemate to resolve. In both cases, their chiral recognition properties and their use in the design of enantiomeric sensing and separation analytical tools will be covered. Enantioselective approaches for larger scale applications, relying for example on immobilized DNA membranes [13-15], will not be addressed here. Finally, the general practical features of the reported DNA- and RNA-based chiral methodologies and some possible perspectives will be discussed.

2. Nonspecific nucleic acid molecules in chiral analysis

2.1. Enantioselective recognition properties

Chirality is an inherent characteristic of the nucleic acid molecules. At the molecular level, the D-ribose or D-deoxyribose subunit possesses numerous asymmetric centres that can be responsible for the enantioselective capabilities of the nucleic acid building blocks. Already in the 1980s, Gil-Av and co-workers demonstrated the ability of some nucleosides (guanosine) and nucleotides (adenosine monophosphate) to discriminate the enantiomers of helicenes in organic solvents [16]. Subsequent studies confirmed the enantioselective recognition ability of these single entities [17, 18]. It was shown by NMR and liquid chromatography that both deoxyribonucleosides and ribonucleosides slightly differentiate the enantiomers of helicenes and other compounds (including propranolol, mandelic acid and benzoin) not only under organic but also hydro-organic [17] and fully aqueous [18] conditions. A more recent work further reported that the uridine triphosphate nucleotide bound to Ag nanoparticles exhibits enantioselective recognition properties towards the enantiomers of cysteine [19].

At a high-order level, the common helix structure of double-stranded DNA (see Fig. 1 for the schematic representation) leads to supramolecular chiral properties, as exemplified by the right-handed (for the common B-DNA form) or the left-handed (for the uncommon Z-DNA form) helical arrangements depending on the ribose conformation [11]. Chaires and co-workers reported the most representative example of the helix handedness impact on the enantioselective binding of chiral DNA ligands: one enantiomer of daunorubicin binds selectively to B-DNA conformation, whereas the other enantiomer prefers the Z-DNA helix [20]. Other nucleic acid motifs such as hairpins, G-quadruplex or three-way junctions (Fig. 1) also display stereoselective capabilities depending on their three-dimensional architectures. Several studies notably described the chiral discrimination properties of single-stranded G-quadruplex motifs towards the enantiomers of both metallo-supramolecular complexes and naphthalenediimide derivatives [21-24]. In parallel, it was demonstrated that the enantiomers of a few amino acids and small organics can be differentiated by stacked G-tetrad columnar structures that originate from the Hoogsteen hydrogen bonding-induced self-assembly of guanosine monophosphate [25, 26]. Moreover, lipophilic guanosine derivatives were successfully used to preferentially extract one dinitrophenyl amino acid enantiomer from water into an immiscible solvent [27]. Brabec et al. also demonstrated that a TWJ motif, formed by the self-assembly of three distinct DNA strands, is able to distinguish the *M* enantiomer from the *P* enantiomer of a chiral tetracationic supramolecular cylinder [28].

As the consequence of the highly polyanionic nature of the phosphate backbone, the aromaticity of nucleobases and the presence of numerous hydrogen bond donors and acceptors, classical ligands of DNA and RNA can interact with these macromolecules through coulombic, π - π stacking and H-bonding forces. The binding regions specifically defined by both the secondary structure and the three-dimensional shape determine in large part the chiral recognition mechanisms of nucleic acid molecules. Different enantioselective modes are then involved in the case of the typical B-DNA double helix [11]. These include (i) minor groove binding for some organic compounds such as distamycin, netropsin, Hoechst 33258 or ofloxacin [29], (ii) intercalation for planar and fused aromatic ring ligands such as daunorubicin or polycyclic aromatic hydrocarbons and (iii) a combination of intercalation and groove binding for metallo-intercalators. As nucleobases can strongly coordinate numerous metal ions, the formation of stereoselective ternary complex between nucleic acid molecules, metal ions and coordination bonding ligands is also effective, as described for the ofloxacin enantiomers with the double-stranded DNA-coordinated Cu (II) transition ion [30]. Binding to other nucleic acid structures could also imply peculiar enantioselective routes in relation to the type of motifs, including for example π - π stacking and side chain interaction with the connection loops for G-quadruplex scaffolds [23, 24] or ligand inclusion into a pre-defined cavity for TWJ [28] or bulged structures (Fig. 1) [8].

2.2. Use of double-stranded molecules

The first use of double-stranded nucleic acids as chiral selectors was accomplished by Streckas and co-workers in 1991 [31]. A B-DNA-based low performance chromatographic column was considered to resolve a few racemates (mixed-ligand diamine-ruthenium(II) complexes) for potential enantiomeric fractionation purposes. The high molecular mass calf-thymus DNA adsorbed onto a hydroxyapatite support acted as the chiral stationary phase and a buffered aqueous solution containing the NaCl salt served as the mobile phase. Later, the more efficient HPLC technique was implemented for the enantiomeric separation of two ruthenium(II) complexes [32]. The chiral stationary phase consisted in the calf thymus DNA macromolecule covalently bound to 5 μ m propylamine-modified silica particles through the periodate oxidization of its extremity followed by a borohydride reduction. Apparent enantioselectivities of about 1.5 were observed under the optimal conditions of mobile phase, *i. e.* acetate buffer containing a small amount of methanol. No further information about the chromatographic performances such as column efficiency and peak asymmetry is available in the paper. Alternatively, a DNA-impregnated paper support was exploited to separate these enantiomers [32]. A similar HPLC assay that used a buffered mobile phase and the calf-thymus

DNA attached to aminopropyl silica ($d_p = 5 \mu\text{m}$) by a carbodiimide-mediated condensation was realized for the separation of the tetrahydropalmatine alkaloid enantiomers [33]. However, the chromatographic data exhibited a significant peak tailing as well as a very poor resolution between the two enantiomers.

Double-stranded DNA molecules were also considered in enantioselective capillary electrophoresis for studying nucleic acid-metal complexes interactions. Either calf-thymus DNA [34] or poly(dA-dT) duplex [35] were dissolved in the background electrolyte (at a concentration in the millimolar range) for the *in situ* formation of diastereomeric complexes under the capillary zone electrophoresis mode. Some enantiomers of chromium(III) and ruthenium(II) metal complexes can be satisfactorily separated in normal polarity conditions by using an uncoated fused-silica capillary. These enantioseparations resulted from not only the differential affinities of enantiomers for the double-stranded DNA molecules and but also the significant difference in the electrophoretic mobility between the free cationic metal complexes and the diastereomeric entities formed with the polyanionic macromolecules.

More recently, shorter double-stranded DNA molecules (of 15 or 23 base pairs) were engineered to conceive chiral fluorescent sensors dedicated to the enantiomeric analysis of tetrahydropalmatine [36]. As depicted in Fig. 2, the assay principle relied on the introduction into the duplex structure of an abasic site, *i. e.* a vacant space within the helix, serving as a stereoselective pocket for the analyte. It was demonstrated that (i) the creation of the abasic site induces a very high enantioselectivity (L/D) of about 100 (*vs* a very low enantioselectivity with the unmodified double-stranded DNA) and (ii) the preferential L-tetrahydropalmatine binding involves π - π stacking interactions with the G bases that flank the abasic site. The sensing platform was based on a kind of label-free indicator displacement assay (Fig. 2). Upon binding to the abasic site, the 2-amino-5,6,7-trimethyl-1,8-naphthyridine fluorophore experienced fluorescence quenching. The addition of the L-enantiomer into the solution containing both the engineered DNA duplex and the fluorophore led to an increase in the fluorescence signal due to the displacement of the bound probe. As the consequence of the stereoselective interaction with the abasic site, a significant difference in the fluorescence change was detected between the L- and D-enantiomers, allowing the purity analysis of samples containing different enantiomeric ratios. The nature of the base opposite to the abasic site and the number of abasic sites along the helix were found to be especially crucial in the sensor sensitivity and enantioselective response. A detection limit for the L-enantiomer of 420 nM was mentioned in the optimal conditions of two separated abasic sites. The general principle was further extended to the

chiral sensing of the thalidomide molecule [36]. In such case, as the consequence of the intrinsic fluorescence properties of this analyte, no fluorescent probe was used to signal the enantioselective binding.

2.3. Use of G-quadruplex molecules and related entities

Besides the double-stranded B-DNA structures, single-stranded motifs such as G-quadruplex elements (Fig. 1) were investigated in some papers for their application in chiral analysis. The pioneering work from the McGown group described an open-tubular electrochromatographic system for distinguishing enantiomers [37]. Two similar single-stranded G-quadruplex structures (including the thrombin aptamer sequence) were covalently attached to the inner surface of the fused silica capillary by using two kinds of linker. The baseline resolution of tryptophan was succeeded in an electric field applied across the functionalized capillary by using a buffer containing 5 mM of K^+ cation, which is known to be essential in the formation of the G-quadruplex motif.

Another study further considered the G-quadruplex structure in the design of an electrochemical sensing platform devoted to the enantiomeric detection of metallo-supramolecular enantiomers. A human telomeric G-quadruplex, 3'-end immobilized onto a gold surface (through an alkyl chain linker) and tagged at its 5'-extremity by a ferrocene label, was employed as chiral selector [38]. In the absence of the enantiomeric ligand, the DNA was unfolded and flexible, allowing close contacts of the redox reporter with the electrode surface and then producing a high current signal. The addition of the *P*-enantiomer triggered the G-quadruplex structuration, constraining the ferrocene away from the surface so that the electrochemical signal was diminished. No signal inhibition was discernible with the *M*-enantiomer, illustrating the enantioselective binding to the immobilized telomeric DNA [38].

Related structures such as G-tetrad helical aggregates, resulting from the self-association of guanosine compounds (see section 2.1), were introduced as a new type of chiral media in capillary electrophoresis [39, 40]. A guanosine monophosphate (10-50 mM) gel, formed in a high concentration of KCl (10-20 mM) required to stabilize the stacked G-tetrad structure, was used to fill the capillary. Under the optimal conditions, the baseline separation of propranolol enantiomers was achieved in less than 5 min with a resolution higher than 2 [39]. Under similar electrophoretic conditions, the chiral selectivity of the guanosine monophosphate gel towards other compounds, the zwitterionic tryptophan and anionic 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate species, was subsequently demonstrated [40]. For the negatively charged compound, electrophoretic resolutions

varying from ~2.5 to ~3.5 and apparent enantioselectivities in the 1.05-1.10 range were measured as a function of the experimental conditions, including changes in the chiral selector and KCl concentrations, solution pH and electromigration parameters. Lower resolutions (1.50-1.70 under the best operating conditions) were obtained with the tryptophan enantiomers. In contrast, the phenylalanine, tyrosine, hydrobenzoin, diprophylline, ketoprofen and flurbiprofen racemates cannot be resolved [40].

2.4. Use of other nucleic acid molecules

Vairamani and co-workers [41, 42] first described the chiral analysis application of nonspecific single-stranded DNA oligonucleotides without any stable secondary structure (Fig. 1). Electrospray ionization mass spectrometry experiments revealed that the very short GCA trinucleotide [41] and a variety of related tetranucleotides [42] distinctly interact with the enantiomers of several natural amino acids. The method relied on the analysis of collision-induced dissociation spectra of the diastereomeric complexes formed between the analytes and the DNA fragments (1:1 or 2:2 adducts were found in relation to the length of oligonucleotides). The chiral recognition ratio was estimated by using the ratio of relative abundance of the precursor ion to that of product ion. This study highlighted that (i) the aromatic amino acids display the higher enantiomer differentiation event, with a chiral recognition ratio ranging from ~3 to ~6 for the most discriminating oligonucleotides and (ii) in many cases, the D-enantiomer of amino acids preferentially binds to the chiral oligonucleotides. The authors suggested that the stereoselectivity could originate from the formation of a loop-like motif induced by the formation of a non-canonical G–A pairing within the short sequence. The approach was finally deployed in the enantiomeric purity analysis of tryptophan [41].

Later, longer single-stranded DNA sequences (from 21 to 24 bases) were investigated for the enantiomer detection by using a field effect transistor device [43]. Oligonucleotides were adsorbed onto single-walled carbon nanotubes by π - π stacking interactions between the DNA bases and the nanotube sidewall. Upon analyte binding to the immobilized nucleic acid molecules, electrostatic changes in the environment of the semiconducting carbon nanotubes can produce a change in the conductance of the sensor. The enantiomers of both the limonene and carvone monoterpenes were strongly discriminated by using three distinct random DNA sequences. For the limonene chiral analysis, a positive conductance change was measured for one enantiomer, whereas a negative response was obtained for the other one. The same group next developed field effect transistor arrays based on the use of such single-stranded DNA-coated carbon nanotubes and reported the chiral

discrimination of limonene and pinene [44]. To the best of our knowledge, these two works represent the only examples of nucleic acid-based chiral sensing under vapor-phase conditions.

More recently, several arbitrarily chosen single-stranded DNA sequences with distinct base compositions, lengths and motifs were evaluated for their chiral discrimination properties in partial-filling capillary electrophoresis [45, 46]. The capillary was filled with a discrete portion of the running buffer containing the UV-absorbing oligonucleotide species, allowing the analytes to be detected without interferences of the quasi-immobile DNA chiral selector zone. More than twenty racemates were injected into the capillary and migrated through the pseudo-stationary phase. It was established that, at a (sub)millimolar concentration, all tested oligonucleotides exhibit some chiral recognition capabilities towards a variety of racemates. In the best cases, the electrophoretic resolution and the theoretical plate number were higher than 4 and 100 000, respectively [46]. The greater chiral discrimination potentialities appear for unstructured (or very weakly structured) oligonucleotides that possess a sufficient chemical diversity. As a representative example, the single 30-mer poly-dA and poly-dT homopolymers displayed a higher range of discriminated racemates than the corresponding poly(dA-dT) duplex (Fig. 3). In addition, two 30-mer heteropolymers, without any stable secondary structure, manifested a larger analyte selectivity spectrum as well as a higher average electrophoretic resolution (~ 2.6 vs ~ 1.5) than the poly-A and poly-T homopolymers, as depicted in Fig. 3 [46]. Among the highly structured DNA motifs, the TWJ element constitutes a special case since thirteen racemates were, at least in part, resolved [45]. This behaviour may result from the presence of the central cavity (Fig. 1) that would promote unique enantioselective interactions *via* an analyte inclusion-type mechanism [28, 47].

All the aforementioned enantioselective sensors and separation methods relying on the nonspecific nucleic acid molecules are listed in Tables I and II.

3. Target-specific nucleic acid molecules in chiral analysis

3.1. Enantioselective recognition properties

Aptamers constitute the major class of small molecule-specific DNA and RNA recognition elements. These single-stranded oligonucleotides are *in vitro* selected for binding to a target by the well-known SELEX process. SELEX is a powerful combinatorial technique that allows the screening of very large oligonucleotide libraries by an iterative process of selection and amplification. Aptamers can interact tightly and specifically with a great variety of species, from metal ions to small molecules, proteins and even cells. The SELEX efficiency allows isolating in many cases stereoselective

aptamers when a small chiral compound is targeted. It has been notably demonstrated by several authors that amino acids [48], drugs [49] or oligopeptides [50] can be strongly enantiodifferentiated by their corresponding specific aptamers. In addition, a counter selection with the non-target enantiomer can be introduced into the SELEX procedure to further enhance the chiral discrimination features. As a representative example, Famulok and co-workers reported an enantioselectivity higher than 12 000 for an L-arginine-binding aptamer by performing a highly stringent selection coupled to efficient counter-selection steps with the D-isomer [51].

The stereoselectivity ability of aptamers very likely comes from their complex three-dimensional shape (mainly hairpins with internal loops, see Fig. 1) and unique binding mechanism. The target recognition typically originates from the adaptability features of nucleic acids. A structural/conformational switch is very often associated to the aptamer-target complex formation through the well-known induced-fit binding mode [52]. In the peculiar case of small molecule binding, the aptameric receptor folds from a more or less disordered structure into an ordered one, which contains a binding pocket that could encapsulate the target. This induced-fit event governs the adjustment of the recognition surface of the aptamer site with the target, defining a great shape complementarity between the two partners through tight contacts between them [52]. In this context, the non-target enantiomer is expected to fit less adequately to the aptamer binding pocket, engaging less stabilizing contacts with the aptamer and/or being submitted to steric hindrance and/or electrostatic repulsion.

3.2. Use in enantioselective separations

Our group reported the first exploitation of aptamers as target-specific selectors in chiral separation [53]. The anti-D-vasopressin hairpin DNA aptamer was initially immobilized through a bioaffinity biotin-streptavidin link onto a chromatographic streptavidin support for use as a chiral stationary phase in micro-LC. The resolution of the oligopeptide was achieved by using a mobile phase containing the Mg^{2+} divalent cation, which had been employed during the SELEX procedure to promote the aptamer active conformation. The very significant chiral discrimination of the aptamer stationary phase was exemplified by the fact that the target D-enantiomer was strongly retained by the column while the non-target L-peptide eluted in the void volume. A poor column efficiency was obtained, as indicated by the reduced plate height h value in the 35-40 range ($h = L/d_p N$ where N is the theoretical plate number, L the column length, and d_p the average particle diameter). This peak broadening was attributed at least in part to slow target-aptamer complex association-dissociation kinetics. The stability of the aptamer column during an extended period of time (≥ 5 months) was

also established [53]. Subsequent works utilized similar approaches for performing the chromatographic enantioseparation of smaller analytes, including adenosine, tyrosinamide and tryptophan [54, 55].

Later, Biostable RNA-based stationary phases were constructed through the mirror-image strategy, *i. e.* by using all-L-RNA aptamers as chiral selectors [56-58]. With a natural D-RNA-based column, the stationary phase was quickly degraded (in only a few days) under usual chromatographic utilization and storage due to the action of naturally occurring RNases. On the other hand, the L-RNA-based column exhibited enhanced stability (≥ 2 -3 months) as the result of their inherent nuclease insensitivity. Due to the chiral inversion principle, the elution order of enantiomers was reversed relative to the D-RNA stationary phase. This mirror-image methodology was implemented for the chromatographic separation of numerous amino acids (arginine, histidine, aromatic amino acids and related compounds) [56-58]. High enantioselectivities were commonly attained, with chromatographic efficiencies similar to that reported with the vasopressin DNA aptamer column.

A 5'-amino-modified aptamer (directed against the D-adenosine enantiomer, see above) was further coupled by the way of an amide bond to 7 μm macroporous carboxylic acid based silica particles [59] in order to design a more robust oligonucleotidic stationary phase relative to those relying on a non-covalent biotin-streptavidin bridge. The covalently bonded stationary phase was found to be stable over the time and under harsh operating conditions (column subjected to a very high 60°C temperature for 6h). It was demonstrated that the enantiomeric separation can be also achieved by using a mobile phase containing a small proportion of organic solvents (until 25% of methanol and 15% of ethanol or isopropanol) [59].

As another interesting application in chiral separation, enantioselective aptamers were exploited as additives in partial-filling capillary electrophoresis [60], following a method similar to that reported in section 2.4. The enantiomeric electrophoretic separation was demonstrated by using a truncated sequence of an anti-D-arginine L-RNA aptamer possessing a pseudoknot motif (see Fig. 1). As the target formed a very stable complex with the chiral selector [50], the D-enantiomer peak can be only detected when the capillary temperature was very high (55°C-60°C). Low apparent efficiency (the theoretical plate number varied from 2 000 to 11 000) and significant peak tailing were observed for the D-arginine target enantiomer. It was suggested that heterogeneous kinetics (due to the presence of different conformers with distinct affinity and kinetic properties) as well as a concentration overload of aptamer binding sites could be responsible for such poor performances [60].

The application range of aptamer-based chiral capillary electrophoresis was subsequently extended

to the separation of anionic racemates [61]. In contrast to cationic analytes, the enantioseparation of negatively charged analytes is assumed to be very difficult to achieve in capillary zone electrophoresis due to the small difference in the electrophoretic mobility between the polyanionic aptamer chiral selector and the anionic compound. In order to overcome this limitation, an aptamer-modified MEKC mode was developed. The principle depended on the use of non-ionic and achiral micelles that act as a pseudostationary phase. A hydrophobic group (cholesteryl)-tagged aptamer can partition into the uncharged micellar phase in such a way the mobility of the aptamer zone was strongly reduced, then promoting the enantioseparation of anionic species (Fig. 4). A PVA-coated capillary was pre-filled with three different zones including a zone containing the immobile non-ionic micellar phase (Tween 20, Brij 35 or Thesit surfactants were successfully used), a zone containing the cholesteryl-tagged anti-adenosine DNA aptamer, and finally a zone containing the adenosine monophosphate (AMP) nucleotide racemate used as model compound. When the electric field was applied, the AMP enantiomers passed through the decelerated micelle-anchored aptamer zone, leading to the chiral separation (Fig. 4). Here again, the method suffered from weak efficiencies (theoretical plate number for the D-AMP target enantiomer ranging from 1 000 to 20 000 in relation to the experimental conditions). The aptamer-modified MEKC mode was further implemented for anionic species of lower mobility than that of the aptameric chiral selector, *i. e.* adenosine diphosphate (ADP) and adenosine triphosphate (ATP), providing the enantioseparation of the three adenine nucleotides in a single run [61].

Finally, Chen and co-workers reported an alternative capillary electrophoresis method, based on mixed chiral discrimination mechanism [62]. The anti-L-tryptophan hairpin DNA aptamer was used in conjunction with sodium cholate micelles, serving as a chiral micellar pseudo-stationary phase, in partial-filling capillary electrophoresis. While the tryptophan racemate cannot be resolved in the presence of either the aptamer or the cholate micelles in the background electrolyte, the combined use of the two chiral selectors allowed achieving the enantioseparation. No further information about the electrophoretic performances can be found in the paper. The method was finally exploited to quantify the D- and L-enantiomers in a test mixture [62].

3.3. Use in enantioselective sensing platforms

The first example of development of an enantioselective sensing platform was described for the chiral analysis of arginine [63]. It was based on the design of a competitive affinity-based capillary electrophoresis assay. A PVA-coated capillary was pre-filled with three different plugs as follows: (i) a plug containing a known amount of the anti-D-arginine L-RNA pseudoknot aptamer (ii) a sample

plug containing the analyte and (iii) a plug containing a fixed amount of a labeled target, *i. e.* D-arginine functionalized by a dansyl group for the UV detection. Upon voltage application, the target first interacted with the aptamer zone, causing the formation of the D-arginine-aptamer complex. Then, the probe passed through the aptamer zone and interacted with the unbound aptamer. The detected tracer free fraction was then enhanced with the target concentration increasing as the result of the fewer available binding sites. A great increase in the probe signal was reported with the D-arginine, whereas the non-target L-arginine did not produce a change in the peak of the free probe. The chiral assay allowed the detection of 0.01% of the D-enantiomer in a non-racemic mixture [63]. Different chiral sensor strategies were further proposed to enantioselectively detect the tyrosinamide analyte. The first reported one was based on a direct fluorescence anisotropy assay by using an anti-L-tyrosinamide 49-mer DNA aptamer tagged at its extremity by the fluorescein fluorophore [64]. Upon target binding, the fluorescently labelled aptamer underwent a sufficiently large conformational transition that hindered the local rotation of the dye, then generating a great increase in the anisotropy response. In optimal conditions, an L-isomer detection limit of 100 nM was reached. No signal change was obtained in the presence of high concentrations of the non-target D-enantiomer, confirming the high enantioselectivity of this aptamer [54]. This allowed the detection of an enantiomeric impurity down to approximately 1% in a non-racemic sample. To note, the same method was successfully extended to the D-AMP chiral sensing by engineering instability in the stem of the anti-D-adenosine hairpin DNA aptamer in order to promote the fluorescence anisotropy response [65]. A homogeneous electrochemical sensor was further implemented for the chiral detection of tyrosinamide [66]. The concept resided in a competitive event between the analyte and a ferrocene-labeled L-tyrosinamide (used as electroactive probe in cyclic voltammetry) for the binding to the anti-L-tyrosinamide aptamer. In the reaction solution containing only the tracer and the aptamer, the electrochemical signal was weak as the result of the slow diffusion rate of probe-aptamer complex. Upon addition of the target to the medium, the electroactive probe was displaced from its complex, leading to an increase in the peak current due to the fast diffusion of the free redox-labelled L-tyrosinamide. The detection of the L-enantiomer at a 0.1% level in a non-racemic mixture was achieved [66]. A fluorescent aptamer-based sensor that permitted the concomitant measurement of both the concentration of each tyrosinamide enantiomer and the enantiomeric impurity in one single sample was next demonstrated [67]. The general principle was based on the chiral inversion principle, as mentioned in section 3.2. As shown in Fig. 5, both D- and L-DNA aptamers, initially tagged with different dyes without spectral overlap (HEX and FAM, respectively),

were present in the reaction solution. In the absence of targets, complementary D- and L-strands functionalized by a quencher formed a duplex structure with their corresponding D- and L-aptamers, leading to a weak fluorescence signal. Upon enantiomer binding to each aptamer, the complementary strands were displaced from their duplexes, then triggering an increase in the fluorescence at two different wavelengths in relation to the D- or L-enantiomer binding. No cross-reactivity between the two reporting mirror-image systems was observed. The enantiopurity analysis method was successfully applied to the monitoring of the yield of D- and L-tyrosinamide during the reaction of D-tyrosine ethyl ester with ammonium hydroxide [67]. To note, Stojanovic and co-workers also conceived a fluorescent assay based on the anti-cocaine TWJ aptamer complementary strand displacement approach in order to differentiate the enantiomers of cocaine [47].

The enantioselective sensing of the vasopressin oligopeptide was conducted through different analytical methodologies using the anti-D-vasopressin DNA aptamer. A label-free indicator displacement assay was designed by using one guanidiniophthalocyanine dye as a fluorescent tracer [68]. The binding of the probe to the internal loop of the anti-D-vasopressin DNA aptamer engendered a fluorescence enhancement. The target interaction with the binding site of the loop provoked the dye release and then generated a decrease in the fluorescence signal. A detection limit of 100 nM was reached for the D-isomer while no significant response was observed with the L-enantiomer. The group next reported a heterogeneous-phase electrochemical D-vasopressin enantioselective sensor that exploits the split aptamer strategy [69]. In such case, the aptamer sequence was split into two DNA fragments that are able to associate only in the presence of the target. One of the two DNA strands, used as capture element, was grafted onto a graphene-mesoporous silica-gold nanoparticle hybrid material, which covered a layer-by-layer assembly of ferrocene-conjugated poly(ethyleneimine) and poly(sodium 4-styrenesulfonate) on the indium tin oxide electrode surface. The addition of both the other DNA fragment and the target enantiomer initiated the formation of the ternary recognition complex that determined a reduction in the electrochemical response of the ferrocene redox probe. Here again, a great stereoselectivity was observed as no signal change was reported with the L-vasopressin. The same team further designed a colorimetric chiral sensor based on the gold nanoparticle aggregation [70]. Due to its di-sulfide bond and guanidinium groups, the vasopressin oligopeptide can adsorb on citrate-coated gold nanoparticles and induce their aggregation, leading to a red-to-purple colour change. When the aptamer was added to the reaction medium, its complexation with the target inhibited the interaction between the D-vasopressin enantiomer and the gold surface, promoting the dispersed state of the

nanoparticles and leading to the purple-to-red colour change of the solution. No significant colour modification was reported in the presence of L-vasopressin. This method was also employed to construct a colorimetric molecular logic gate system [70]. Finally, the vasopressin enantioselective sensing was very recently accomplished by using an orthogonal detection scheme [71], along the lines described above for the simultaneous detection of tyrosinamide enantiomers (Fig. 5). In such case, the complementary strand displacement strategy was replaced by the aptamer kissing complex approach, for which the target-bound folded state of a hairpin aptamer can be recognized by a hairpin probe *via* a loop-loop interaction [72]. The fluorescence anisotropy technique was utilized as the signalling method by tagging the D- and L-hairpin probes with two different dyes (fluorescein and Texas Red) without spectral overlap. The aptamer kissing complex-based sensor provided the concomitant quantification of the two enantiomers in a single solution as well as the enantiomeric impurity detection at a level as low as 0.01%.

The chiral detection of a drug, *i. e.* ibuprofen, was also described through a colorimetric transduction approach [73]. DNA aptamers, directed against either S- or R-ibuprofen, were immobilized onto gold nanoparticles, preserving them from the salt-induced aggregation. The binding of the target enantiomer to its aptamer led to the displacement of the oligonucleotide from the gold surface and then induced the red-to-purple colour change dependent on the dispersion-aggregation switch of the nanoparticles. Detection limits in the nanomolar range were achieved for the S- or R-ibuprofen enantiomers (using their corresponding specific aptamers) through visible absorption spectroscopy measurements. The non-target enantiomer did not display colorimetric response, demonstrating the very high chiral recognition properties of the used oligonucleotides. The detection was subsequently achieved under a simpler format involving the implementation of a smartphone apparatus [73].

Finally, another kind of functional nucleic acids was exploited to develop a fluorescence anisotropy sensor for the enantiomeric determination of L-histidine [74]. This was based on the use of *in vitro* selected DNAzymes that possess the ability to cleave a single (ribo)phosphodiester bond of a substrate module in the presence of its specific cofactor. The sensor consisted in an enzyme module that triggered the L-histidine cofactor-dependent cleavage of a RNA phosphodiester bond of a fluorescently labelled substrate domain. In the absence of the cofactor, the anisotropy signal was significant due to the large molecular mass of the complete enzyme-substrate structure. Upon addition of L-histidine, the substrate splitting at the cleavage site determined the dissociation of single strands of lower size, inducing a decrease in the fluorescence anisotropy response. A limit of detection of 0.6 μM can be reached for the L-enantiomer. No change in the anisotropy signal was

observed with D-histidine, revealing that the cross-reactivity of the DNAzyme with the non-target enantiomer was negligible. The method was able to signal the presence of trace amounts of the minor enantiomer (0.05%) in a non-racemic mixture [74].

Tables I and II summarize the different chiral separation and sensing methods based on target-specific nucleic acid molecules.

4. Practical considerations and perspectives

All the works listed in this review clearly highlight that one of the major issues of the nucleic acid-based chiral selectors is related to their restricted analyte spectrum. For the nonspecific nucleic acid molecules, the developed chiral methodologies based on structured double-stranded DNA (and to a lesser extent G-quadruplex) molecules are mainly limited to very peculiar high-affinity chiral binders such as transition metal complexes or metallo-supramolecular assemblies (Tables I and II). The use of single-stranded DNA without stable secondary structures [41-44, 46] or high-order molecules containing a pre-defined binding pocket (TWJ, helix with abasic sites, bulged structures for example) [36, 45] could overwhelm at least in part these drawbacks and expand the selectivity range, as discussed above (Tables I and II). As compared with the double helix structure, it would seem that the unstacked and/or unpaired state of bases could facilitate the accessibility of potential interaction sites. The exposed nucleotides of these molecules would then more easily accommodate the binding of low-affinity chiral ligands and then enlarge the enantioselectivity range. Moreover, Helene et al. [75] and Shen et al. [76] have demonstrated several years ago that weak DNA binders such as aromatic amino acids and norfloxacin interact more strongly with the denatured DNA than with the classical double helix. Nevertheless, these species remain far to compete with the standard chiral selectors of very broad analyte spectrum such as polysaccharides, synthetic polymers and small molecular receptors [1-5]. Concerning the target-specific oligonucleotides, a set of aptamers directed against different enantiomeric compounds (amino acids, oligopeptides and nucleosides/nucleotides) have been successfully used at the analytical level (Tables I and II). Although somewhat comparable in terms of analyte range with other existing target-specific chiral selectors such as molecular imprinted polymers [77] or antibodies [78], no more than fifteen analytes have been yet enantiodifferentiated and only three chiral analytical methods dedicated to the very important drug class have been described at the present time (Tables I and II).

To date, the reported nucleic acid-based enantiodiscrimination systems have been only achieved in liquid phase, except for two examples related to the design of chiral field transistor devices [43, 44].

In many studies, an aqueous solution containing mono- and/or divalent metal cations is required in order to maintain the secondary structure and the active three-dimensional shape of the nucleic acid motifs. As a representative example, the chromatographic separation of adenosine enantiomers using an aptamer chiral stationary phase is completely abolished when the column is flushed with a buffered mobile phase containing EDTA [59]. The addition of a modest amount of organic modifiers is however feasible, at least in some cases [57, 59]. In separation sciences and notably in chromatography, this peculiar criterion constitutes a strong disadvantage over some aforementioned chiral selectors that are compatible with various HPLC modes (normal-phase, reversed-phase, polar-organic) and different other techniques (SFC, GC), then **limiting** the diversity of chiral recognition mechanisms, the method flexibility and the analyte applicability.

It is well established that the reaction temperature plays an important role in the stereospecific binding to chiral selectors and the temperature increase generally triggers a significant reduction in the apparent enantioselectivity as the result of the common enthalpically driven chiral recognition mechanism [1]. In the nucleic acid-based chiral analysis, the temperature factor is peculiarly critical as it may influence not only the enantiomer-receptor interactions but also the structure and conformation of the nucleic acid molecules in a complex fashion [53, 79]. For sophisticated motifs, multiple conformers with heterogeneous binding properties can even be present within a relatively narrow temperature window [59]. In addition, working at a temperature beyond the fusion temperature results in the loss of the functional motif for structured nucleic acids. It is however important to notify that the denaturation is reversible upon return to a lower temperature range. All these considerations imply that the reaction temperature has to be very carefully controlled for developing the nucleic acid-based chiral methods, both in the separation and sensor areas.

In the reported chiral methods, high apparent enantioselectivities are frequently retrieved especially with the target-specific chiral selectors for which the non-target enantiomer often exhibit low (or negligible) response, *i. e.* retention/migration shift or signal change in separation techniques or sensors, respectively [36, 53, 56, 60, 64, 66-74]. These strong discrimination features, coupled to efficient transduction techniques, are particularly attractive in the development of sensitive chiral sensors for the enantiomeric determination at trace levels (in the 0.01-0.1% range). On the other hand, in separation sciences, the significant enantioselective properties of the chiral selector may cause quantification problems if the non-interacting enantiomer elutes (or migrates) as a single peak with matrix interfering compounds [53, 56]. Furthermore, the enantiospecific binding to nucleic

acids is often associated to slow association-dissociation kinetics that can be detrimental in separation, leading to a poor-to-very poor chromatographic/electrophoretic efficiency [53, 56, 60].

In terms of stability over time, the DNA chiral selectors commonly exhibit satisfactory features, at least under buffered aqueous conditions. For example, there is commonly no significant changes in the analyte retention and enantioselectivity for several months when DNA-based chromatographic columns are operated under standard conditions of use and storage [53, 59]. The great instability of RNA selectors, rapidly degraded by RNases, can be overwhelmed through the implementation of the mirror-image strategy [56-58]. **This approach consists in the use of L-oligonucleotides (sometimes referred to as spiegelmers) that are mirror-image of “natural” D-oligonucleotides. As the structure of nucleases is inherently chiral, the RNases accept only a substrate in the correct chiral configuration, *i. e.* the D-RNA species, while the L-RNA molecule is unsusceptible to the naturally occurring enzymes.** L-nucleic acid molecules could be also advantageously exploited for chiral analysis in biological environments as they greatly limit the matrix interferences originating from sample proteins [80]. However, this is ensured at the expense of the synthesis cost, the L-oligonucleotides being much more expensive than the natural ones.

Considering the general conditions of use and applicability, the nucleic acid chiral selector class appears to closely resemble that of the proteins, which moreover also include both nonspecific (bovine serum albumin, α 1-acid glycoprotein, cellobiohydrolase I etc...) [1] and target-specific (antibodies) [78] enantiodiscriminating elements. **Protein chiral selectors suffer from a number of limitations that include the risk of irreversible denaturation and the lack of batch-to-batch reproducibility. In addition, proteins are naturally occurring polymers available in only one chiral configuration. This implies that the detected enantiomer in sensing devices or the enantiomer retention/migration order in separation cannot be controlled. In contrast, the synthetic nature of oligonucleotides provides higher reproducibility and allows targeting one analyte enantiomer in relation to the chosen chiral configuration of the selector. Furthermore, as mentioned above, the nucleic acid denaturation is a reversible process.** Finally, owing to their unique properties of programmability, predictability and ease of manipulation, nucleic acids offer several unique sensor schemes based not only on the strand displacement or assembly strategies (see Table II) but also on various other potential ones [81].

Despite the different achievements discussed in the current review, the use of nucleic acids chiral selectors in analytical sciences remains in its early stages and very substantial efforts are still needed to develop enantioselective tools of broader applicability and real practicability. **Although several**

companies devoted to DNA-based molecular assays have emerged during the last decade (for example, BasePair Biotechnologies, NeoVentures Biotechnology, Novaptech, Somalogic in the aptamer field), no enantioselective sensing or separation systems have been yet commercialized. We can envision that an improvement in the range of discriminated enantiomers could be attained through the implementation of other enantiospecific binding modes and techniques. For example, in aqueous solution, the ligand-exchange mechanism using metal ions in conjunction with nucleic acids as chiral selecting species is expected to induce new interesting chiral recognition properties for a variety of important low-affinity analytes, as suggested by a previous study [30]. In addition, along the lines reported for the nucleoside/nucleotide chiral selectors [16, 17], working under organic solution conditions could be investigated with surface-grafted (in chromatography or heterogeneous-phase sensors) single-stranded nucleic acids for which the chiral recognition interactions are known to not depend on structured motifs. One could even imagine that these unstructured single-stranded molecules, when immobilized on chromatographic supports, would exhibit some chiral recognition properties under gas [43, 44] or supercritical fluid phase conditions for potential GC or SFC applications.

Other important progresses in the chiral resolution capabilities can originate from the extension of the chemical diversity space of the nucleic acid molecules. The incorporation of a variety of commercially available base/backbone chemical substituents (hydrophobic, cationic for example) could greatly enlarge both the enantioselective mechanisms and the analyte spectrum, as widely demonstrated with other types of chiral selectors [1-5]. For the target-specific chiral selectors, an additional enhancement of the targeted racemate range could be achieved through the implementation of improved SELEX procedures based on non-natural nucleic acid libraries [49].

Finally, mechanistic studies on the chiral recognition properties of nucleic acid-based chiral selectors constitute another important field to investigate in the future. Notably, only a very few data on the molecular basis of the enantioselective binding to single-stranded DNA (or RNA) molecules are available [53, 79, 82] and deep structural and computational studies are required to more clearly understand the origins of their enantiodifferentiation ability.

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Table I: Summary of the described nucleic acid-based enantiomeric separations

| | Chiral selectors | Enantiodifferentiated analytes | Analytical methods | Ref. |
|--------------------------------------|--|---|--|--------------|
| Nonspecific nucleic acids | Calf-thymus DNA | Ruthenium(II) metal complexes | Low-performance LC | [31] |
| | Calf-thymus DNA | Ruthenium(II) metal complexes | HPLC | [32] |
| | Calf-thymus DNA | Ruthenium(II) metal complexes | Paper-based chromatography | [32] |
| | Calf-thymus DNA | Tetrahydropalmatine | HPLC | [33] |
| | Calf-thymus DNA, poly(dA-dT) duplex | Ruthenium(II) and chromium(III) metal complexes | CE | [34, 35] |
| | Single-stranded G-quadruplex | Tryptophan | Open-tubular CEC | [37] |
| | Guanosine monophosphate self-assembly | Tryptophan, propranolol, 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate | CE | [39, 40] |
| | Single-stranded DNA sequences (~15 oligonucleotides) | Aromatic amino acids, drugs, illegal drugs, nucleosides (~20 racemates) | Partial-filling CE | [45, 46] |
| Target-specific nucleic acids | DNA hairpin aptamer | Vasopressin | Micro-LC | [53] |
| | DNA hairpin aptamer | Adenosine | Micro-LC | [54, 59] |
| | DNA aptamer (unknown structure) | Tyrosinamide | Micro-LC | [54] |
| | L-RNA hairpin aptamer | Arginine | Micro-LC | [56] |
| | L-RNA hairpin aptamer | Tyrosine, tryptophan, DOPA and some derivatives | Micro-LC | [57] |
| | L-RNA hairpin aptamer | Histidine | Micro-LC | [58] |
| | L-RNA pseudoknot aptamer | Arginine | Partial filling CE | [60] |
| | DNA hairpin aptamer | AMP, ADP, ATP | Partial filling MEKC | [61] |
| | DNA hairpin aptamer | Tryptophan | Low-performance LC Partial filling MEKC | [55] [62] |

Table II: Summary of the described nucleic acid-based enantiomeric sensing platforms

| | Chiral selectors | Enantiodifferentiated analytes | Analytical methods | Ref. |
|---|--|---|---|-------------|
| Nonspecific nucleic acids | Duplex DNA containing an abasic site | Tetrahydropalmitine | Fluorescent indicator displacement | [36] |
| | Telomeric G-quadruplex | Metallo-supramolecular helicates | Direct electrochemistry | [38] |
| | Single-stranded unstructured DNA oligonucleotides (3 to 4 bases) | Amino acids (~15 racemates) | Electrospray-mass spectrometry | [41, 42] |
| | Single-stranded unstructured DNA oligonucleotides (21 to 24 bases) | Limonene, carvone, pinene | Field effect transistor | [43, 44] |
| Target-specific nucleic acids | L-RNA pseudoknot aptamer | Arginine | Competitive affinity-CE with UV detection | [63] |
| | DNA aptamer (unknown structure) | Tyrosinamide | Direct fluorescence anisotropy | [64] |
| | | | Competitive electrochemistry | [66] |
| | D- and L-DNA aptamers (unknown structure) | | Complementary strand displacement with fluorescence detection | [67] |
| | DNA hairpin aptamer | Adenosine monophosphate | Direct fluorescence anisotropy | [65] |
| | TWJ DNA aptamer | Cocaine | Complementary strand displacement with fluorescence detection | [47] |
| | DNA hairpin aptamer | Vasopressin | Fluorescent indicator displacement | [68] |
| | | | Split aptamer-based electrochemistry | [69] |
| | | Gold nanoparticle colorimetric detection | [70] | |
| D- and L-DNA hairpin aptamers | | Kissing complex-based fluorescence anisotropy | [71] | |
| DNA aptamer (secondary structure not studied) | Ibuprofen | Gold nanoparticle colorimetric detection | [73] | |
| DNAzyme | Histidine | Fluorescence anisotropy | [74] | |

Figure 1. Schematic representation of common nucleic acid sequences/motifs retrieved in the chiral discrimination literature. **(a)** unstructured single-stranded element, **(b)** double-stranded motif, **(c)** double-stranded motif with bulge, **(d)** hairpin, **(e)** hairpin with internal loop, **(f)** G-quadruplex, **(g)** Three-way junction, **(h)** pseudoknot.

Figure 2. General sensing scheme of a fluorescent indicator displacement chiral assay based on an engineered DNA duplex containing an abasic site as enantioselective pocket for the tetrahydropalmatine analyte [36].

Figure 3. Enantiomeric separations in partial-filling capillary electrophoresis by using different nonspecific DNA molecules as chiral pseudo-stationary phases [46]: **(a)** 30-base pair poly(dA-dT) duplex (left) and the corresponding 30-base poly-dA (middle) or poly-dT (right) homopolymers and **(b)** two 30-base unstructured heteropolymers. Operating conditions: [DNA] = 1.60 mM, DNA zone in the capillary ~40 cm.

Abbreviations of enantioseparated compounds: ISP: isoprenaline, TRA: tramadol, 1-Nal: 1-naphthylalanine, PL: propranolol, VPM: verapamil, Qal(2'): H- β -(2-quinolyl)-Ala-OH, CQ: chloroquine, Trp: tryptophan, 1-MT: 1-methyl-tryptophan, Bta: H- β -(3-benzothienyl)-Ala-OH, MDMA: methylenedioxy-N-methamphetamine, dA: 2'-deoxyadenosine, NE: norepinephrine, dG: 2'-deoxyguanosine, PMA: paramethoxyamphetamine, WME: tryptophan methyl ester.

Figure 4. **(a)** Schematic principle of the aptamer-based micellar electrokinetic capillary electrophoresis mode [61]. **(b)** Resolution of the D, L adenosine monophosphate (AMP) racemate using Brij35 non-ionic micelles (surfactant concentration = 25 mM) in the background electrolyte and a zone of cholesteryl-tagged anti-D-AMP DNA aptamer (200 μ M) as chiral selector.

Figure 5. General sensing scheme of the simultaneous orthogonal fluorescent detection of both tyrosinamide enantiomers in a single sample using the complementary strand displacement strategy [67]. DNA elements: aptamer (in black) and complementary strand (in red); dyes: HEX (hexachlorofluorescein) and FAM (fluorescein).

Figure 1

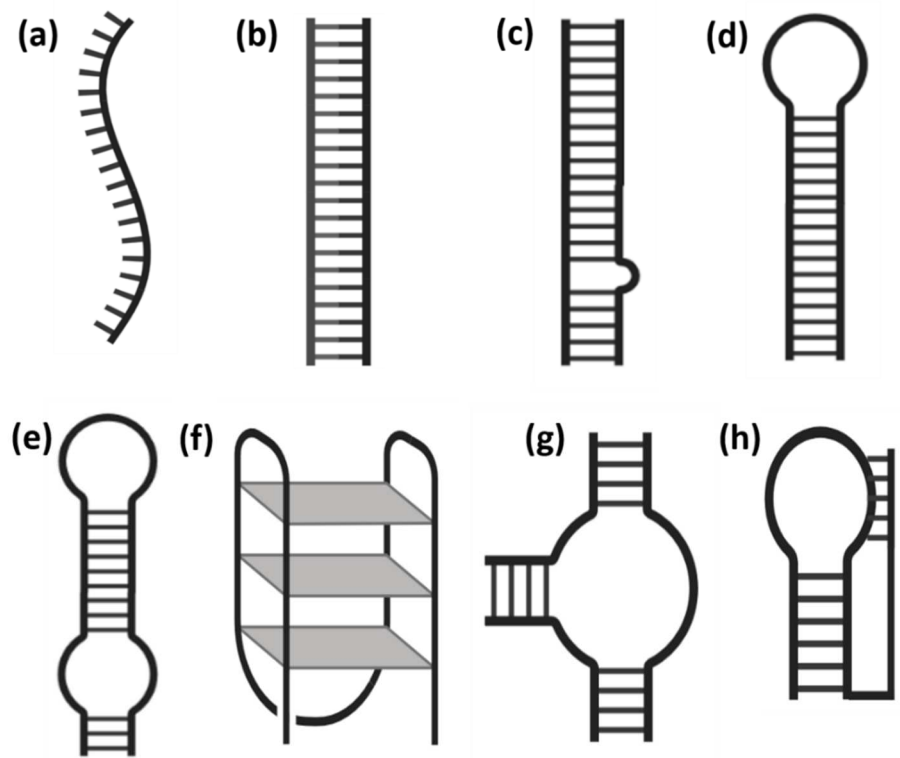


Figure 2

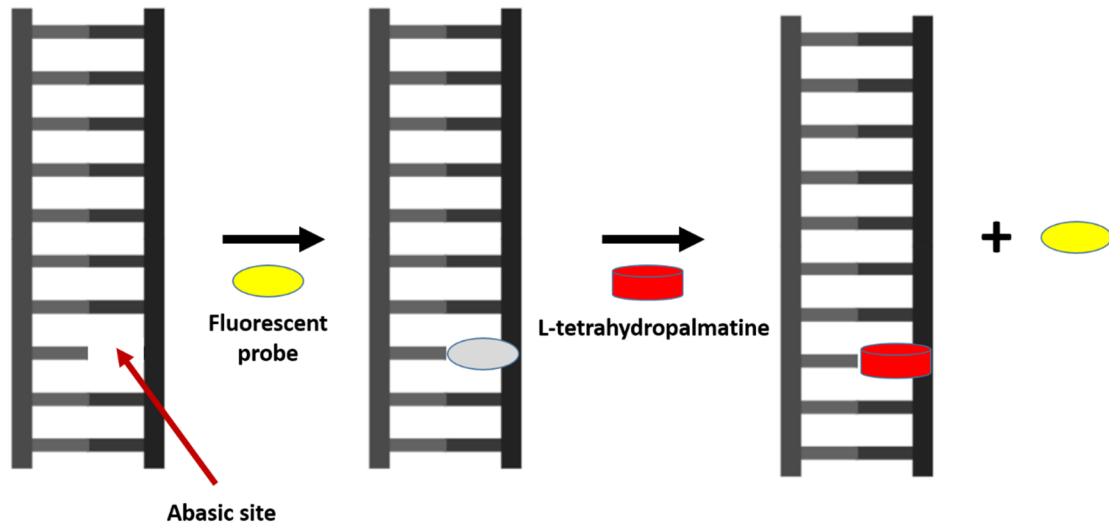
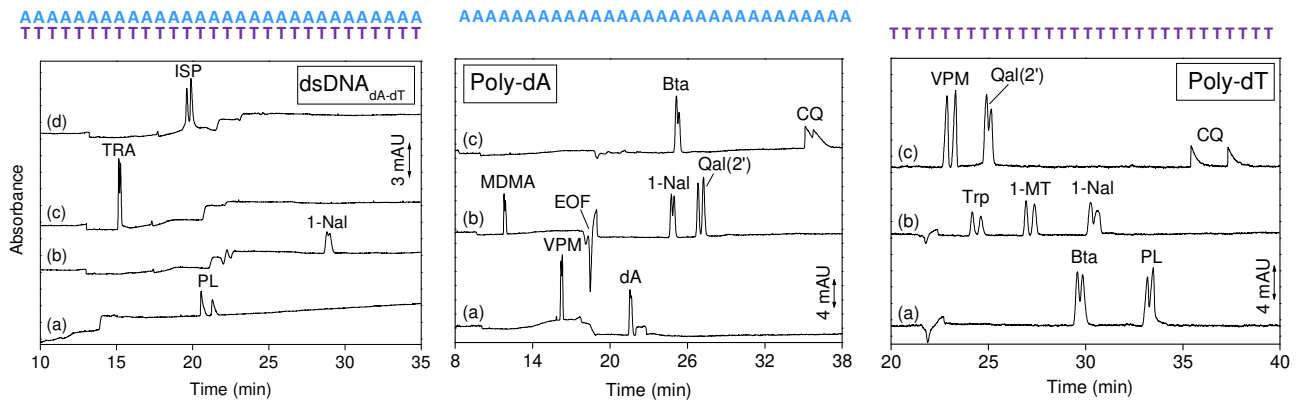


Figure 3

a)



b)

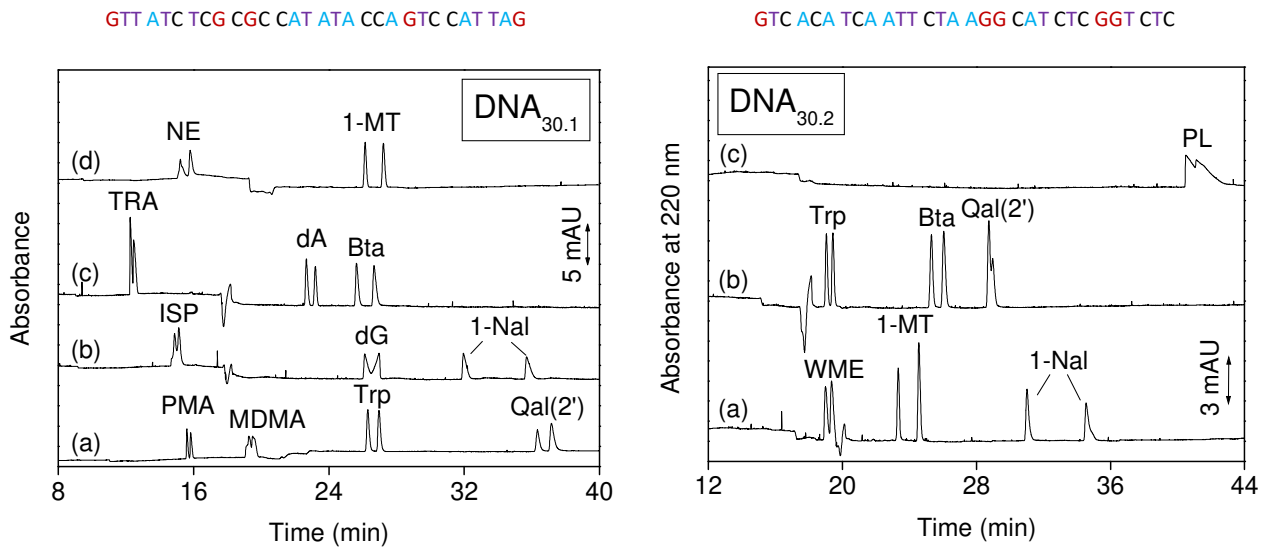
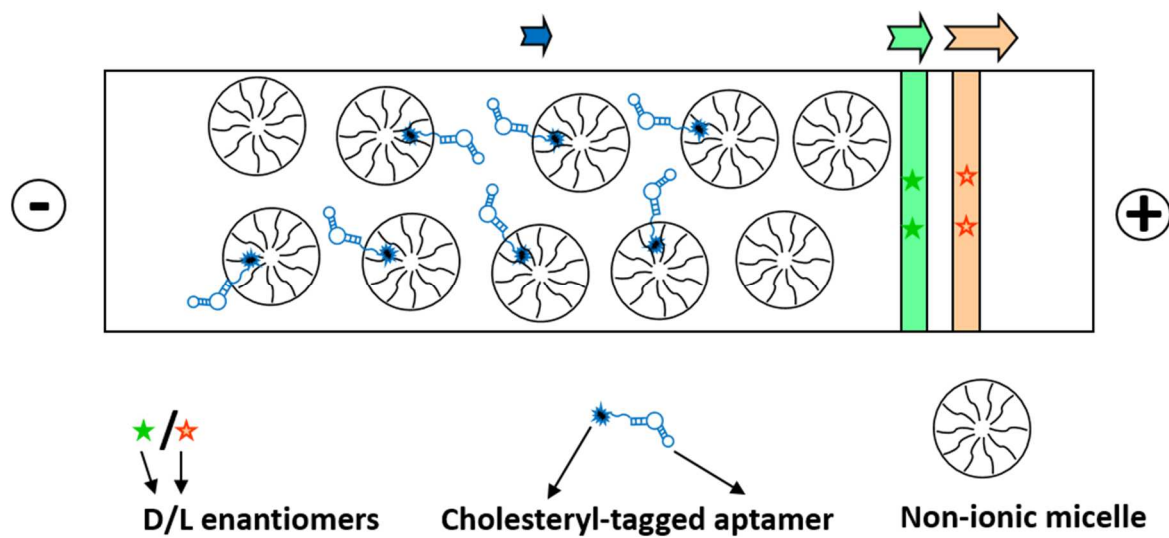


Figure 4

a)



b)

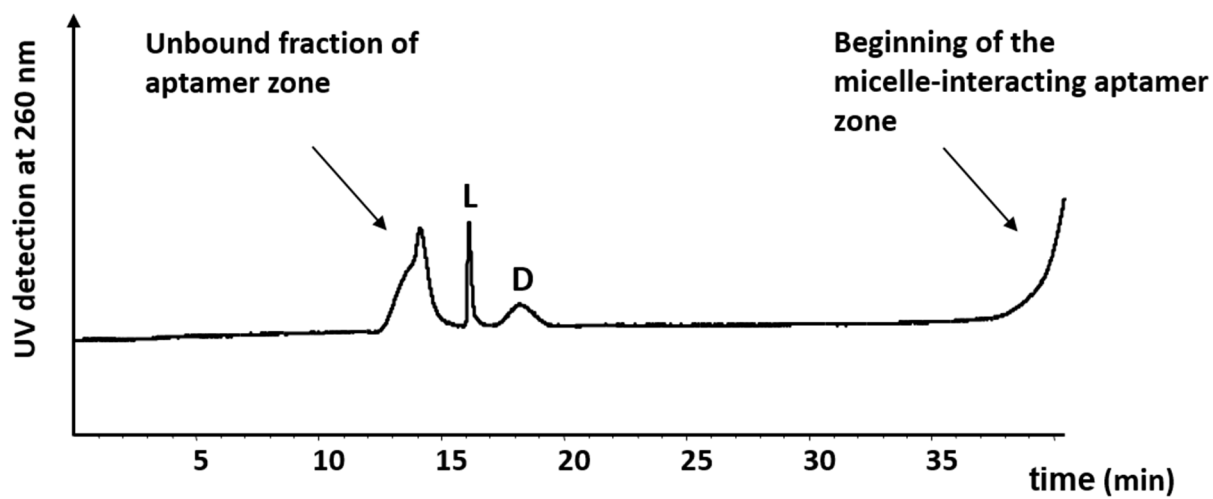
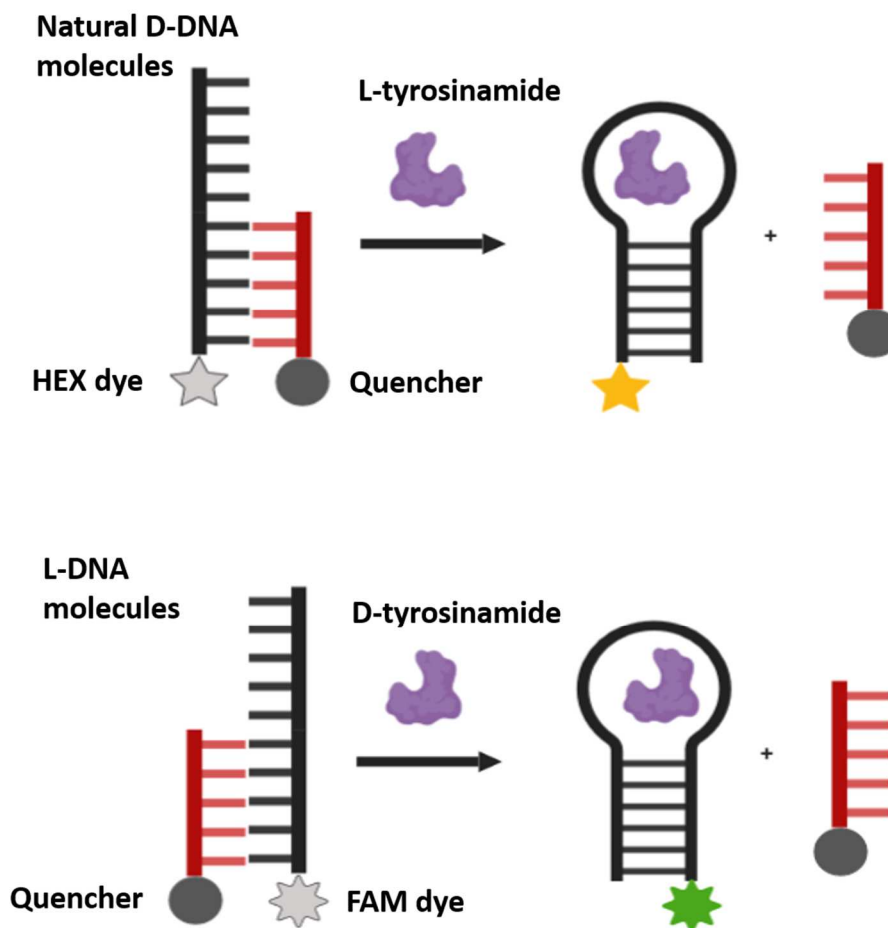
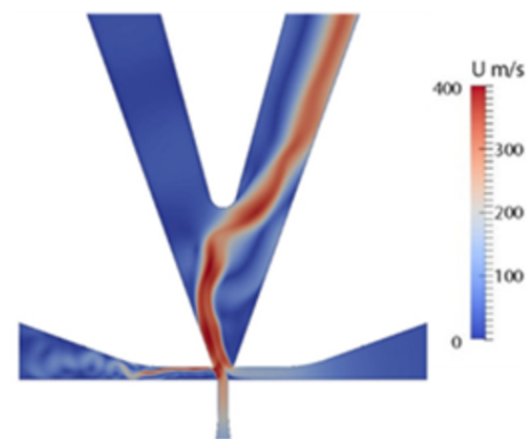
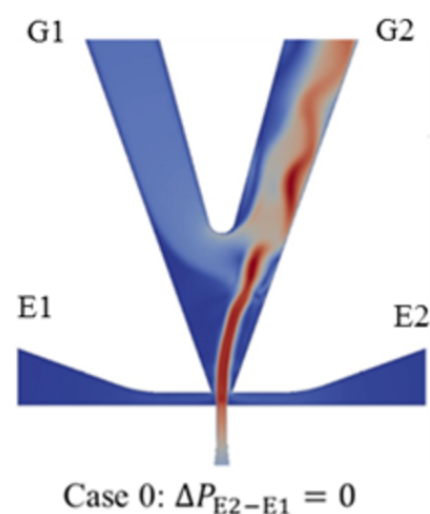
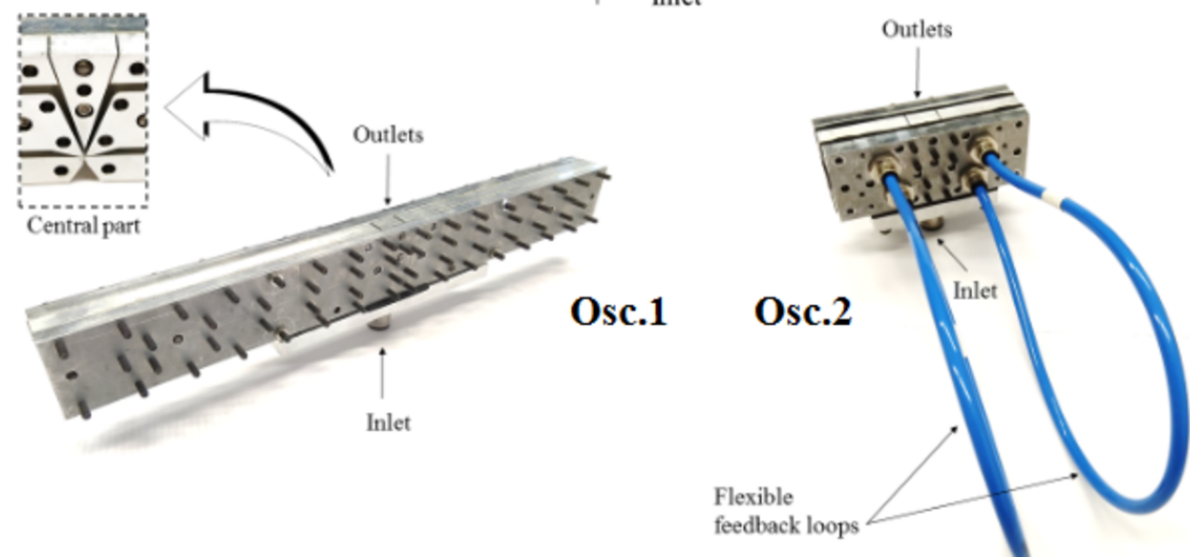
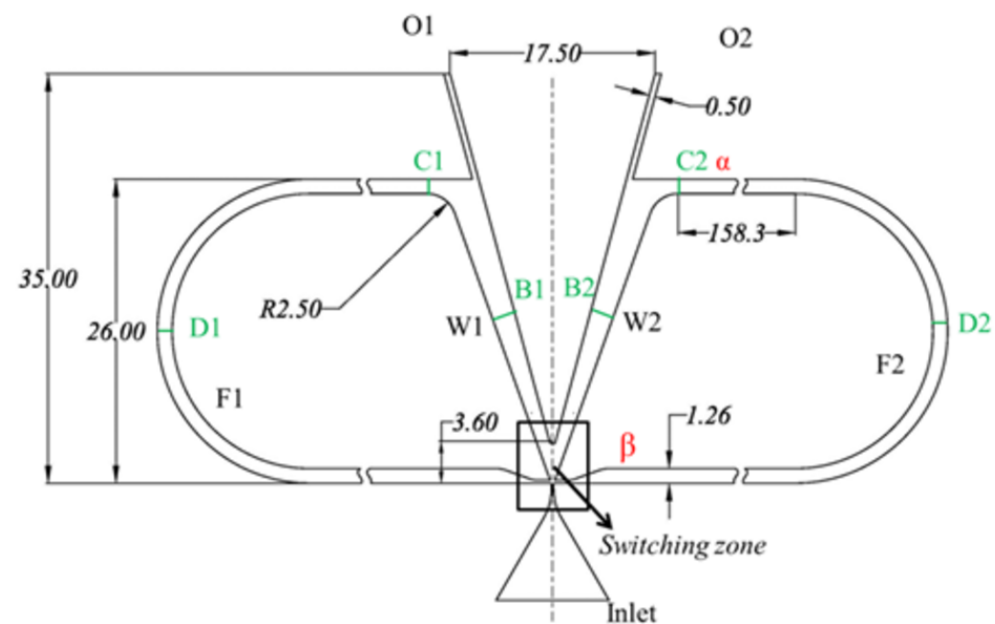


Figure 5





Case 1-5: $\Delta P_{E2-E1} = 0.4P_i$

