

This item is the archived peer-reviewed author-version of:

Mapping the gaps in chemical analysis for the characterisation of aptamer-target interactions

Reference:

Daems Elise, Moro Giulia, De Almeida Campos Rui, De Wael Karolien.- Mapping the gaps in chemical analysis for the characterisation of aptamer-target interactions
Trends in analytical chemistry - ISSN 0165-9936 - 142(2021), 116311
Full text (Publisher's DOI): <https://doi.org/10.1016/J.TRAC.2021.116311>
To cite this reference: <https://hdl.handle.net/10067/1794070151162165141>

Mapping the gaps in chemical analysis for the characterisation of aptamer-target interactions

Elise Daems,^{1,2,3‡} Giulia Moro,^{1,3,4‡} Rui Campos,^{1,3} and Karolien De Wael^{1,3*}

¹AXES Research group, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

²BAMS Research group, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

³Nanolab Centre of Excellence, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

⁴LSE research group, Università Ca' Foscari of Venice, Via Torino 155, 30172 Mestre, Italy

‡ These authors contributed equally to this work.

* Corresponding author.

Abstract

Aptamers are promising biorecognition elements with a wide applicability from therapeutics to biosensing. However, to successfully use these biomolecules, a complete characterisation of their binding performance in the presence of the target is crucial. Several multi-analytical approaches have been reported including techniques to describe kinetic and thermodynamic aspects of the aptamer-target interaction, and techniques which allow an in-depth understanding of the aptamer-target structures. Recent literature shows the need of a critical data interpretation, a combination of characterisation techniques and suggests the key-role of the characterisation protocol design. Indeed, the final application of the aptamer should be considered before choosing the characterisation method. All the limitations and capabilities of the analytical tools in use for aptamer characterisation should be taken into account. Here, we present a critical overview of the current methods and multi-analytical approaches to study aptamer-target binding, aiming to provide researchers with guidelines for the design of characterisation protocols.

Keywords: aptamer, DNA, aptamer-target binding, analytical characterisation, aptasensor

Abbreviations:

Adenosine triphosphate (ATP), bilayer interferometry (BLI), circular dichroism (CD), deoxyribonucleic acid (DNA), electron microscopy (EM), electrospray ionisation (ESI), enthalpy variation (ΔH), entropy variation (ΔS), fluorescence anisotropy (FA), fluorescence polarization (FP), Gibbs free energy variation (ΔG), ion mobility (IM), isothermal titration calorimetry (ITC), binding constant (K_D), kinetic isothermal titration calorimetry (kinITC), loop-mediated isothermal amplification (LAMP), molecular dynamics (MD), mass spectrometry (MS), microscale thermophoresis (MST), mass-to-charge ratio (m/z), nuclear magnetic resonance (NMR), quartz crystal microbalance (QCM), rotating droplet electrochemistry (RDE), ribonucleic acid (RNA), small-angle X-ray scattering (SAXS), systematic evolution of ligands by exponential enrichment (SELEX), surface enhanced Raman scattering (SERS), serial femtosecond crystallography (SFX), surface plasmon resonance (SPR), surface plasmon resonance imaging (SPRi).

1. Introduction

Aptamers are short deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) single strands with a specific three-dimensional conformation, or fold, that are able to selectively bind a given target. Aptamers can vary in length (20-100 nucleotides) and can have various structural motifs, such as stems, loops, hairpins, bulges and pseudoknots [1]. The concept of aptamers was developed in the beginning of the 1990's in the labs of Gold [2] and Szostak [3,4]. Aptamers are selected from large pools of randomised oligonucleotide sequences by a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [2,5]. Compared to other traditional molecular biorecognition elements such as antibodies or enzymes, aptamers offer considerable advantages. First, they can be designed for a wide range of possible targets, going from small molecules to tumour cells [6,7]. Second, they are a synthetic chemical product rather than a biological one. This brings forth the ease of modification, high reproducibility and purity and a low production cost. Furthermore, aptamer sequences are not immunogenic since they are generally not targeted by the immune system [6]. Finally, they are stable over a broader temperature and pH window and have a longer shelf-life compared to antibodies or enzymes [8].

In the last 25 years, the number of publications dealing with aptamers and their applications has increased exponentially as shown in Figure 1. Nowadays aptamers are largely applied in therapeutics [6,9], diagnostics [10], drug delivery [11,12] and in sensing devices as biorecognition elements [13–15]. There were also developments in the selection process for aptamers and multiple variations exist, such as FluMag SELEX [16] which is nowadays the most commonly used one, capture-SELEX [17] developed specifically for small molecules and graphene oxide SELEX [18] that does not require immobilization of aptamer, or target, in contrast to other SELEX approaches.

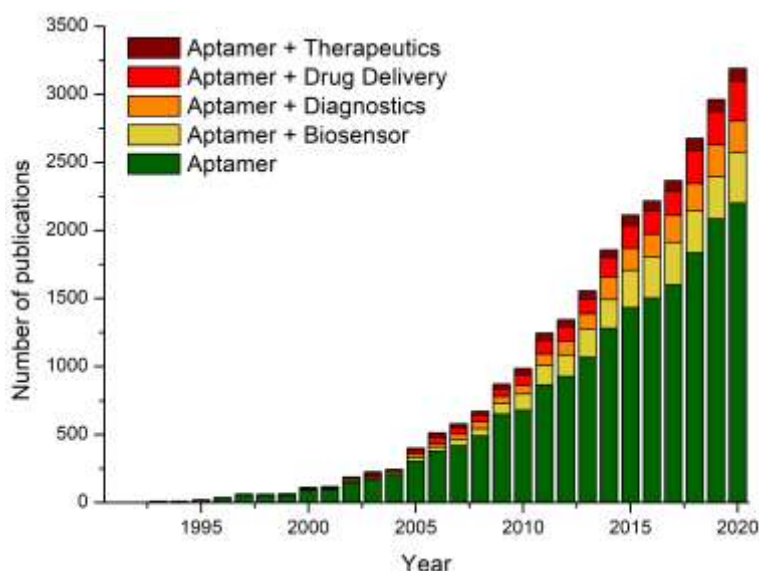


Figure1 – Number of publications per year related to aptamers and their applications. The plot shows the exponential growth of the publications in the field over the last 15 years. Bibliographic analysis performed with Scopus searching for the following keywords: “aptamer*”, “aptamer* AND biosensor*”, “aptamer* AND therapeutic*”, “aptamer* AND drug delivery*” and “aptamer* AND diagnostic*” per year. Data from Scopus retrieved on 14/01/2021.

Despite the advances in SELEX procedures and the variety of aptamers applications, examples of non-functioning aptamers (unable to bind their target) were recently reported in literature, showcasing the key-role of a complete characterisation of the aptamer-target interactions [19–21]. Zong and Liu [19] reported an

arsenic(III)-binding aptamer, which has been used in more than two dozen peer-reviewed articles, and showed that there was no specific binding. In 2020, Bottari *et al.* [20] and Tao *et al.* [21] described the lack of binding of a previously reported ampicillin and chloramphenicol aptamer, respectively. These studies were all based on multi-analytical approaches involving complementary techniques ranging from isothermal titration calorimetry (ITC) to native mass spectrometry (MS). The results emphasise the need for a thorough characterisation of aptamers before proceeding to their application. This need for a critical evaluation of aptamer performance was also claimed during the 2019 meeting of the international society on aptamers, in Oxford (UK). Furthermore, McKeague *et al.* [22] found that the use of certain techniques can enhance or reduce the affinity of a given aptamer, indicating the need for multiple characterisation strategies and a critical choice of these methods depending on the final application.

Here, we provide a critical overview of analytical techniques that can be used for the characterisation of aptamer binding and structure. We focus on newer analytical methods rather than classical methods (e.g. equilibrium dialysis, chromatography approaches and gel-based methods) that can be used for studying aptamers. These classical methods were previously discussed by Mirando-Castro *et al.* [23]. First, techniques to retrieve kinetic and thermodynamic information on the binding event are discussed. A distinction is made between techniques used in solution and on a confined surface since immobilisation can alter the affinity [24,25]. Moreover, label-free methods and analytical protocols in which a label (such as a fluorophore) is required are addressed separately. Second, we focus on techniques providing information on the aptamer and aptamer-target structures. This part is divided in high- and low-resolution techniques which can provide structural information.

2. Kinetic and thermodynamic studies of the binding event

Determining the extent at which the aptamer-target interaction occurs represents a fundamental step of aptamer characterisation. Kinetic and thermodynamic parameters help in the decision of choosing an aptamer over another. Yang *et al.* [26] selected aptamers for steroids based on the higher affinity towards the target even though other sequences were also specifically binding to the target.

The analytical methods used in this frame can be divided in two main clusters: methods in which interactions are studied in solution (section 2.1) and methods in which aptamer-target kinetics are characterised at a confined surface after immobilization of one of the binding partners (section 2.2).

2.1 In solution

Several analytical techniques can provide kinetic or thermodynamic information on the aptamer-target interaction. When studying the interaction in solution, there are two possibilities: using label-free methods (section 2.1.1) or methods in which either the aptamer, the target, or both carry a label (section 2.1.2).

2.1.1 Label-free methods

Isothermal titration calorimetry and related methods

ITC, first described by Wiseman *et al.* [27], directly measures the heat exchange of biochemical reactions, or molecular interactions, at constant temperature. Experiments are performed by titrating a target into a solution containing the aptamer and the heat which is released or absorbed after each addition is monitored [28]. ITC is regarded as a standard characterisation method which enables drawing a detailed thermodynamic picture of aptamer-target molecular interactions not only for small-molecules but also for cell targets [29,30]. From the ITC thermogram, and its corresponding binding curve, it is possible to obtain information on the binding constant (K_D) and the reaction stoichiometry.

Philip Johnson's group has extensively used ITC to study the cocaine binding aptamer [31–34]. On the most recent work [34] they considered the changes in the Gibbs free energy (ΔG) to study the (de)stabilising effect of dangling nucleotides on the aptamer-target complex stability. To validate the possibility to relate these structural features with the variation of a thermodynamic parameter, such as ΔG , the authors used the adenosine triphosphate (ATP) aptamer as a model system. This approach underlined the importance of using appropriate control experiments, such as using a model system and negative controls, depending on the design of the experiment to aid ITC data interpretation.

Despite its potential, ITC can be easily subjected to misinterpretation (*i.e.* non-appropriate fitting). An example of that is the difference in K_D values reported by Amato *et al.* [35], using ITC, and by Zavyalova *et al.* [36], using a turbidimetric assay, when studying the RA36 oligonucleotide which consists of two repeats of the thrombin-binding aptamer. The discrepancy was investigated by Antipova *et al.* [37] and their findings supported the theory of Zavyalova *et al.* that RA36 has two binding sites with a different affinity. Therefore, the difference in K_D of the original papers could be explained by the use of a one-set-of-site binding model instead of a two-stage binding process. This shows that the method to fit the experimental data is of great importance for correct ITC results [38]. The fitting will be optimal if the so-called *c*-value (which is equal to $nK_a[A]$, wherein *n* is the number of binding sites per aptamer *A*), ranges between 10 and 500. It is, however, not always possible to work in this window of *c*-values, particularly for low affinity systems. In case the aptamer concentration cannot be increased to obtain *c*-values in this range, fixing the binding stoichiometry (*N*) during data analysis can minimize the error for experiments performed at low *c*-values. Another approach could be the use of displacement methods in which a higher affinity target that binds competitively with the target of interest is used [39,40]. These examples show that ITC is a powerful technique that should be handled with care, otherwise it may lead to conclusions that do not reflect the processes taking place.

Additional insights about the binding mechanism can be acquired by performing ITC measurements at different temperatures and considering the enthalpy variation (ΔH) vs. temperature, *i.e.*, by following the changes in the heat capacity. Amano *et al.* [41] ascribed the negative heat capacity observed for the aptamer-target complex formed between the high-affinity RNA aptamer and its target protein to the burying of a large hydrophobic region upon binding. Thermodynamic parameters (ΔH and entropy variation (ΔS)) were also used to determine the structure switching mechanism of cocaine aptamers upon binding to the target [42]. Zhang *et al.* [43] used the adenosine aptamer, that contains multiple binding sites, to show that the combination of rational sequence design and ITC analysis allows the characterisation of each binding site separately and the comparison of their thermodynamic behaviour. Also a one-site aptamer was developed for adenosine, using ITC to prove the 1:1 binding model and test it towards possible interfering compounds normally interacting with the corresponding wild type aptamer [44].

Conventional ITC is suitable for studying aptamer-target interactions with a K_D in the nM - μ M-range [45]. For systems which interact more strongly, it becomes unreliable due to the large variation in injection heat when the molar ratio of aptamer/target is only increased slightly. This problem can be overcome by using ligand-displacement ITC as first proposed by Sigurskjold [45]. Kuo *et al.* were later able to use this method to determine a K_D in the pM-range for a streptavidin-binding aptamer [46].

Apart from providing a complete thermodynamic characterisation, ITC apparatus and new processing methods allow to directly analyse association kinetics from raw ITC data. Burnouf *et al.* [47] developed a new processing method named *kinetic ITC* (kinITC) in which the shape of each injection peak is analysed to obtain kinetic information. This can be applied to a large variety of systems from folding to ligand binding. ITC-based kinetics

was first applied to long timescale reactions aiming to avoid complications related to the time needed for heat flow detection in the calorimeter cell. The optimisation of ITC empirical response model enables now to follow reactions occurring in the order of ten seconds [48]. A kinITC method was incorporated in, for example, the AFFINImeter software to obtain a full thermodynamic and kinetic description of one-step interaction systems [49] (a complete introduction can be found in [50]). Despite these recent advances, the applicability of kinetic-based ITC to aptamer-target systems is still limited and the majority of the examples are related to enzymatic systems (i.e. to tackle inhibitors binding [51]).

Native mass spectrometry

Native electrospray ionisation mass spectrometry (ESI-MS) allows the characterisation of non-covalent complexes, such as aptamer-target complexes, due to the transfer of the sample from solution to the gas-phase using a soft ionisation method and the use of gentle experimental conditions during the analysis [52]. Native MS delivers information about the stoichiometry of the binding (given that the K_D is in the nM to low μ M range) and allows the simultaneous identification and characterisation of multiple species, which are co-existing in equilibrium (e.g. aptamer, target and complex), based on the mass-to-charge ratio (m/z) of each species [53].

Native MS was employed as a fast screening technique to determine the potential binding of natural molecules to a thrombin-binding aptamer folded into a G-quadruplex structure [54]. The screening allowed to detect the formation and/or the coexistence of complexes with different stoichiometries, 1:1 and 1:2. Moreover, by performing competition experiments the authors indirectly proved stacking interactions between one of the alkaloid targets and the G-tetrads. Marchand *et al.* showed that also the enthalpic and entropic contributions to the formation of a complex can be identified using a temperature-controlled nanoelectrospray source (Figure 2) [55]. Using G-quadruplexes as an example, insights into the driving forces behind target binding could be obtained by looking at the thermodynamic signatures. Gülbakan *et al.* determined the binding selectivity and stoichiometry of three small molecule-binding aptamers and also showed that native MS can provide accurate aptamer-target binding affinities (by incorporating the correct controls) and can help to refine the model for aptamer-target binding equilibrium [56].

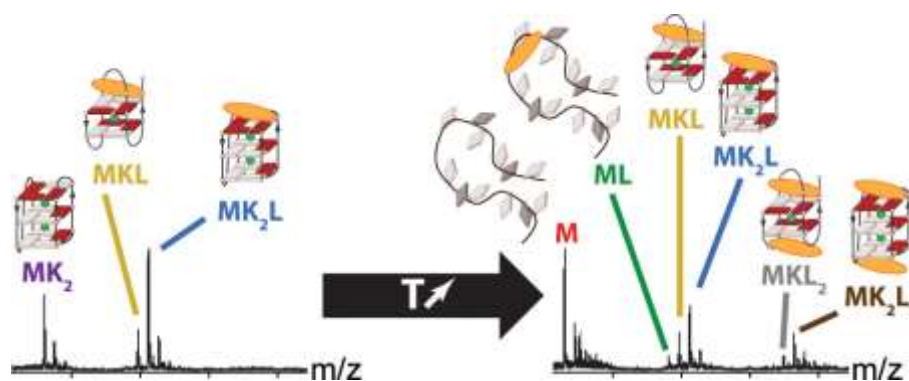


Figure 2 – Native MS was used to detect and quantify each specific stoichiometry: number of strands in the G-quadruplex, number of potassium ions (in green) and number of ligands (in yellow). By using a temperature-controlled electrospray source, the behaviour of the complex at different temperatures was monitored. From this, temperature-dependent equilibrium constants and thus the enthalpic and entropic contributions to the formation of each stoichiometry were determined. Reprinted with permission from [55]. Copyright (2018) American Chemical Society.

A careful experimental design is needed when performing native MS experiments to ensure non-denaturing conditions. Porrini *et al.* showed that duplexes up to 36 base pairs undergo compaction in the gas-phase due to phosphate group self-solvation prevailing over Coulomb repulsion [57]. However, Daems *et al.* [58]

demonstrated that key-features of aptamers and their targets are preserved in the gas-phase. The authors used a set of related cocaine-binding aptamers, displaying a range of folding properties and ligand binding affinities and showed that their apparent quinine-binding affinity, qualitatively matched the previously reported K_D values obtained by ITC [59–61] as shown in Figure 3. By combining native MS with ion mobility (IM) spectrometry, which provides information on the size and shape of ions, the authors found differences in IM-behaviour between the free aptamer and aptamer-quinine complexes. These differences were attributed to a small target-induced conformational changes and were found to inversely correlate with the affinity of binding. This MS-based approach provided a fast way to study the aptamer-target recognition.

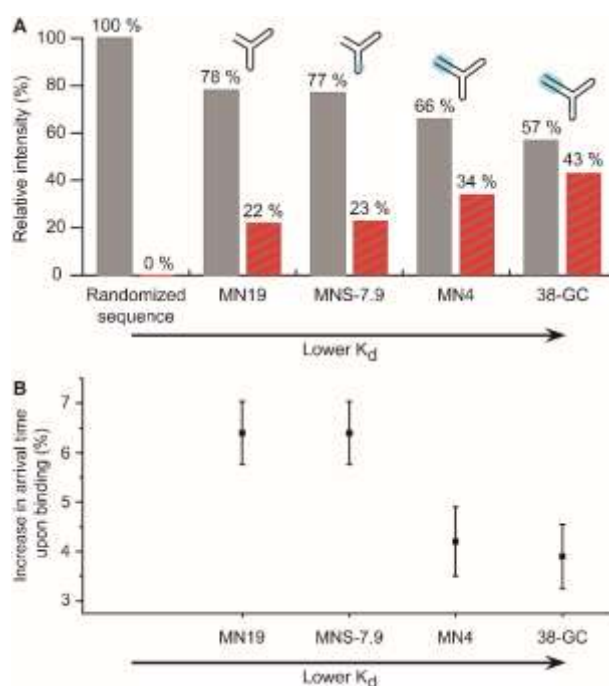


Figure 3 – Native IM-MS measurements of four cocaine-binding aptamers (MN19, MNS-7.9, MN4, and 38-GC) and a randomized sequence in complex with quinine. A) The relative abundance of the aptamer and complex are shown in grey and red, respectively. B) Changes in IM-behaviour, i.e. the arrival time, for all aptamers. More rigid aptamers (MN4 and 38-GC) were found to undergo smaller conformational changes than flexible aptamers (MN19 and MNS-7.9). Reprinted with permission from [58]. Copyright (2021) Elsevier.

Electrochemical methods

To avoid covalent labelling, redox probes and rotating droplet electrochemistry (RDE) methods can be employed. The kinetic RDE setup developed by Limoges and co-workers, which is depicted in Figure 4, allowed to i) work in hydrodynamic conditions (avoiding slow diffusion limitations), ii) have a real-time monitoring and iii) assure a rapid mixing of the reactants in the microliter droplet [62]. Challier *et al.* [29] combined RDE with fluorescence polarization (FP), ITC and quartz crystal microbalance (QCM) to characterise the binding process of L-tyrosinamide to its aptamer, highlighted in Figure 4. The authors found that the interaction occurs via an induced-fit mechanism in which the L-tyrosinamide first binds to the 5'-end of the aptamer after which the aptamer folds around the target.

Redox-active molecules were also used in other approaches. Guyon *et al.* [63] proposed a displacement strategy in which non-redox-active aminoglycosides bind to an RNA riboswitch, or 16S RNA, and displace a redox-active target. This increases the diffusion rate of the redox-active target and leads to an enhancement of the electrochemical signal. Paromomycin was found to be an efficient redox spy probe to study the binding of several aminoglycosides. Also loop-mediated isothermal amplification (LAMP) has been used in combination with aptamers and redox-active molecules [64,65]. Xie *et al.* [64] developed an electrochemical method for

the detection of Ochratoxin A. In this approach, a situation in presence and absence of the target is compared. In absence of the target, the aptamer can undergo amplification during the LAMP reaction resulting in a high amount of dsDNA, which is then able to capture the intercalating methylene blue in solution. The aptamer-target complex is, however, less prone to undergo amplification and as a result free methylene blue is present in solution which can undergo the electrochemical reaction and results in a signal. Recently, Martin *et al.* [66] offered an interesting overview of intercalating and non-intercalating redox probes compatible with dsDNA (such as osmium complexes, methylene blue and derivatives, Nile blue, etc.) by comparing their performance upon the same LAMP reaction. They concluded that intercalating probes result in a higher LAMP sensitivity.

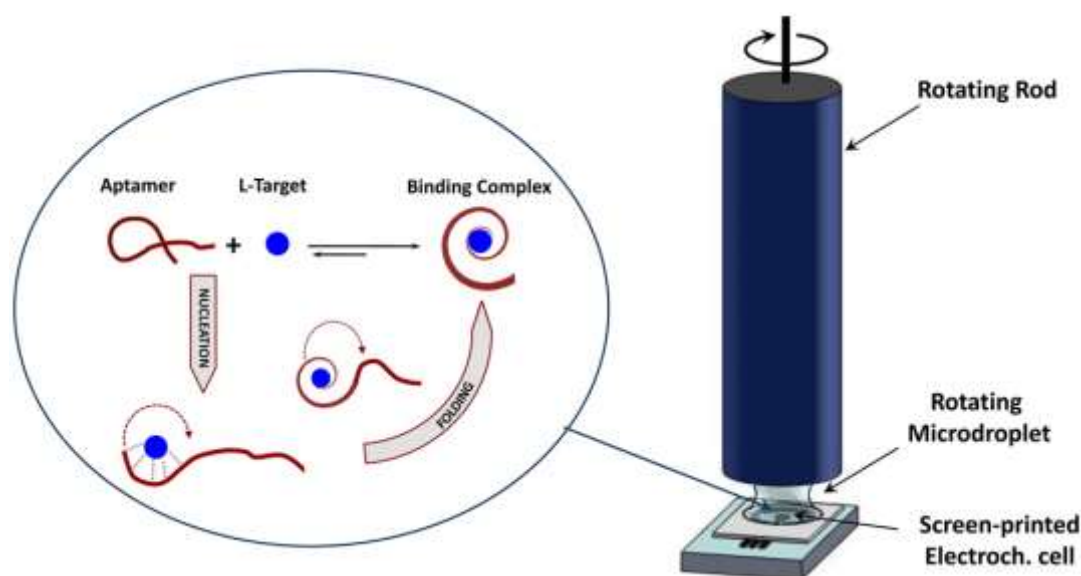


Figure 4 – Scheme of the kinetic rotating droplet electrochemistry (RDE) setup developed by Limoges using a screen-printed three-electrode electrochemical microcell (with a carbon working electrode, a silver reference electrode and a carbon counter-electrode), on the right. Aptamer-target interaction processes investigated with the RDE method, on the left. Reprinted with permission from [29]. Copyright (2016) American Chemical Society.

Fluorescent-based methods

In case the aptamer or target has intrinsic fluorescent properties, these can be used to characterise the aptamer-target interaction in solution although only a few recent examples are available in the literature. Jaeger *et al.* [67] used the intrinsic fluorescence of ciprofloxacin to study its binding to an RNA aptamer selected for this target. The fluorescence of ciprofloxacin is quenched upon binding to the aptamer and the change in intensity can be correlated to the amount of target bound. This allowed the authors to determine the K_d of the aptamer-target complex. Moreover, by using derivatives of ciprofloxacin the influence of different side groups was investigated. Fadock *et al.* [68] used an ochratoxin A aptamer with fluorescent nucleobase analogues at different positions to study the folding and binding. A fluorescent signal is observed upon binding of the target and this can be used to measure the aptamer affinity. Furthermore, since certain probe locations hampered binding of the target, insights in the binding location were obtained.

2.1.2 Labelled methods

Aptamer-target binding kinetics can be characterised in solution also with the help of labelled-based strategies. Once one of the binding partners is labelled with a fluorophore or a quencher, the aptamer-target interaction is indirectly monitored by the changes in fluorescence. These strategies allow estimating aptamer-target kinetics and they are often combined within versatile methods. Additional information regarding the binding mechanism can be acquired by modifying the label or its position (i.e. labelling a different region of the aptamer). In general, the presence of labels may affect the aptamer-target binding process thus labelled-

based aptamer-target characterisations require to be supported by other techniques or a complete series of negative controls. The extensive optimisation often required for labelled-based strategies makes them more suitable for the characterisation of aptamer-target complexes of interest for assays or sensing applications than extensive aptamer screening.

Fluorescent-based methods

Well-known for cellular and tissue imaging, fluorophores have been extensively used to label biomolecular complexes, such as protein-protein and DNA/RNA-protein complexes [69], and to map their interactions even at a single nucleotide level [70]. Once a fluorophore is covalently attached to one of the binding partners, aptamer-target interactions can be monitored by following the changes in the fluorescent quantum yield, through quenching phenomena, fluorescence anisotropy/polarization (FA/FP), etc.

Among these techniques, FA is commonly used in aptamer-target characterisation methods [71]. FA records the polarised fluorescence emission of the label in horizontal and vertical directions under polarised excitation light and correlates its rotation speed to the fluorescence lifetime. The changes in anisotropy/polarization reflect the changes in the mobility of the fluorophore (*i.e.* local rotation or molecular volume), for instance, by the structure-switching of the labelled aptamer upon target binding.

Initially limited to macromolecule-aptamer characterisation, FP/FA methods are now also designed for studying aptamers for small-molecules thanks to, for example, molecular mass amplifying strategies [72] or nanomaterial-based enhancement [73,74]. In these methods, the nature and position of the fluorophore label plays a key-role, as exemplified in the study for an aptamer-based FA assay for immunoglobulin E reported by Zhao *et al.* [71]. To optimise the assay, the authors compared a series of aptamers, which differed only in the position of the fluorescein label, Figure 5. Depending on the location of the fluorescein on the DNA sequence, the K_D was significantly influenced. This also allowed to discriminate close-contact sites and identify the strong affinity binding region. An accurate identification of this region required a large series of experiments in which the target is tested in presence of truncated or modified aptamers, as previously shown in the study of aptamer probes for B1 with tetramethyl rhodamine label [75]. In both examples, the authors underlined that their studies were conceived to be further applied in the design of methods and aptasensors.

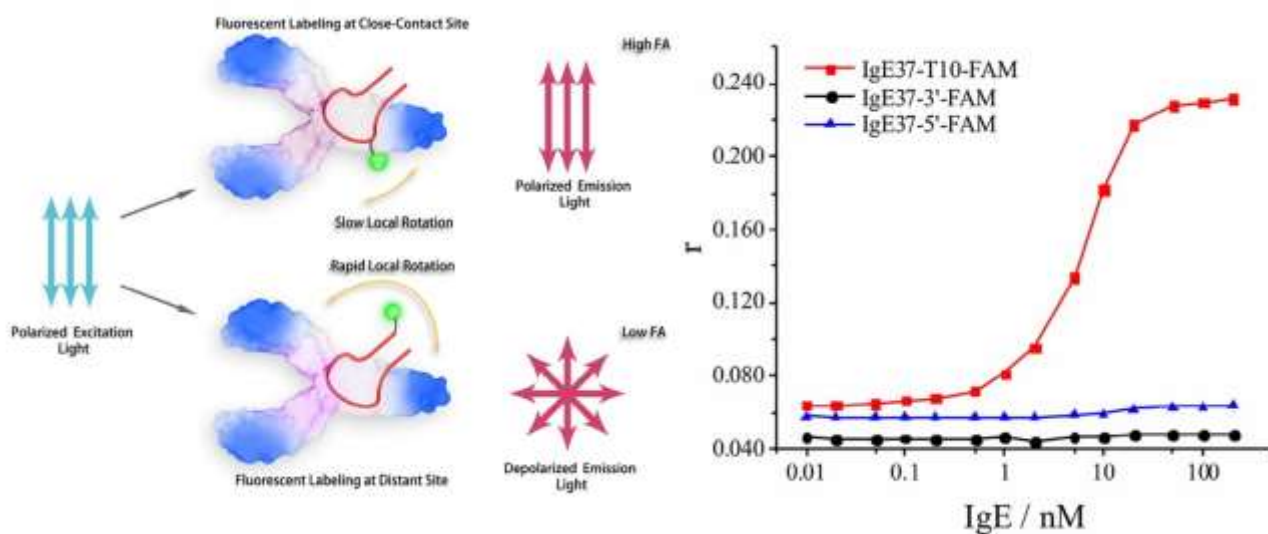


Figure 5 – Fluorescence anisotropy analysis of IgE and its aptamer with a single fluorescein label (FAM). The site of the label influences the movement of the label and therefore also the light emitted. Slow local rotations lead to emission of polarised light, while rapid

rotations lead to depolarised emission. The results show the label at the T10 position can detect binding of the aptamer to IgE, while the aptamer with label at the 3' or 5' end does not detect the binding. Reprinted with permission from [71]. Copyright (2020) Elsevier.

Indeed, fluorescent-based methods in general are not suitable for simple characterisation or screening of aptamer-target kinetics and should be evaluated in consideration to the final aptamer-target application. To optimise the design of the labelled aptamers, a structural characterisation by X-ray crystallography can be included, as previously done for fluorescent-labelled RNA aptamers used in transcript tracking [76]. By using a fluorophore-labelled aptamer and a quencher-labelled complementary sequence, Yang *et al.* investigated the cross-reactivity of aptamers for steroids [26]. In the presence of a steroid which binds to the aptamer, a fluorescent signal is observed. By comparing the amount of fluorescence observed, the affinity of multiple targets could be distinguished. Fluorophore labels are often combined also with heterogenous methods. The transition from *in-solution* to *at a confined surface* (section 2.2) might affect the aptamer performance at a different extent, as discussed by Amaya-González *et al.* [77]. In that work, the authors reported a comparison between biotin and fluorescein labelling showing that fluorescent labels are not always the best choice. Therefore, it is suggested to consider other labelling strategies when relatively high and unreliable K_D values are obtained with fluorophore labels.

Microscale thermophoresis

Another method in which the fluorescence of a labelled molecule is detected, is microscale thermophoresis (MST). MST is based on the movement of molecules through temperature gradients, the so-called thermophoresis. This movement, which is detected by fluorescence, depends on the size, charge, and hydration shell of an aptamer and at least one of these parameters changes upon binding of a target [78]. Typically, MST is used to determine the binding affinity of an aptamer-target system in which either the target [79] or the aptamer [80,81] is labelled. The technique can, however, also provide some other insights when using multiple aptamers or targets. Rubio *et al.* [82] showed that two aptamers for the protein β -conglutinin bind to different sites of the proteins using a competitive MST assay. Furthermore, Skouridou *et al.* [83] investigated a progesterone aptamer and range of steroids with small structural differences. The affinity of the aptamer against all steroids was determined using MST and by overlaying this information with the structures of the steroids, the moieties involved in binding could be identified. MST might not be able to detect binding if the aptamer does not undergo significant conformational changes as shown by Prante *et al.* [84] using an 25-hydroxivitamin D aptamer. This aptamer did not show any response at room temperature but at 35 °C, which is closer to the melting temperature of the aptamer, a binding curve could be obtained. This is due to the fact that the aptamer adopts a less structured state at elevated temperatures and folds into its native form upon addition of the target and allows detection using MST.

2.2 At a confined surface

The first report of an aptamer being used in a biosensor dates back to end of the 20th century when Hieftje *et al.* showed that aptamers could be used in biosensing by immobilising a fluorescently labelled anti-thrombin DNA aptamer on a glass support and selectively detecting thrombin in solution [85]. Nowadays aptamer immobilisation protocols are commonly used in the design of optical, electrochemical or mass-sensitive aptasensing strategies [25,86–88]. Covalent immobilization protocols were reported requiring to modify the aptamers with specific linkers (chemical functionalities, such as thiols) [89,90], but also non-covalent methods resulted successful, like the electrostatic interaction-based one described by Farjami *et al.* [91].

Despite its covalent or non-covalent nature, the immobilisation process can influence the aptamer-target binding. Potryailo *et al.* reported beneficial effects in terms of affinity [85], while other researchers observed a drop in the affinity constants when working at confined surfaces and this drop was ascribed to steric

hindrance effects related to the proximity of the aptamer to the surface of interest [24,25]. Also the nature and length of the linker/spacer used for covalent immobilisation was found to influence the affinity [24,77,92]. Increasing the spacer length generally results in a more accessible aptamer and a better affinity towards the target, but too long spacers can reduce the affinity due to a smaller surface density of the aptamer or interaction of the spacer with the aptamer resulting in an incorrect fold [24,92].

Once at a surface, the aptamer-target binding can be characterised through different methods, which are mostly applied in aptasensing and here briefly though critically discussed. For completeness, even techniques which are not providing binding information, in terms of affinity and conformational changes, are mentioned to emphasise the difference in between binding characterisation (qualitative and/or quantitative information about the aptamer-target binding) and binding monitoring (verification of the occurrence of the binding).

2.2.1 Optical methods

Optical detection methods are commonly used in aptasensing strategies to monitor the binding of a target to its aptamer but to a lesser extent for the characterisation of aptamer-target kinetic behaviour. Optical sensing platforms can rely on, *e.g.*, fluorescence or colorimetric assays [93], surface-enhanced Raman scattering (SERS) [94], surface plasmon resonance (SPR) [95,96] and bio-layer interferometry (BLI) [97]. Since optical methods are usually used for sensing purposes and not for characterisation of aptamers and their complexes, only few examples of optical-based strategies will be presented.

Surface plasmon resonance

SPR has been extensively used as a label-free method to detect aptamer-target interactions at a confined surface providing information on kinetics and affinity depending on the setup design [98,99]. In general, SPR platforms include a transducing element (planar metal chip), which interrelates the biochemical and optical domain allowing to monitor the changes in reflectivity, and an electronic component [100,101]. The variation in the refractive index (reflectivity) reflects the changes in mass concentration at the chip surface, which is related to the binding event. In a conventional SPR experiment, the reflectivity recorded in resonance units is measured at a fixed angle and wavelength to assure the optimum sensitivity towards the selected aptamer-target complex.

The adaptability of these platforms depends on the possibility to immobilise one of the interacting partners (aptamer or target) on the sensor surface and add the other one in solution. In case the target is a small molecule (< 1000 daltons), it is usually the target that is immobilised while the aptamer is in solution to obtain a detectable variation in reflectivity. Once the architecture is optimised, a wide range of binding conditions can be screened as showed by Minagawa *et al.* in the selection and characterisation of a salivary α -amylase aptamer [102]. After measuring the K_D of the candidate sequences, the authors studied the binding specificity of the one with higher affinity by testing it in presence of other related salivary biomarkers. Importantly, the binding affinities determined via SPR platforms cannot be directly compared with these obtained with other techniques, as clearly showed by the dataset presented by Sass *et al.* [81]. Here, the K_D for an anthracycline aptamer and a truncated form calculated via SPR was two orders of magnitude higher than that determined using MST, which is a labelled immobilisation-free strategy. This example underlines how the immobilisation step can influence the binding and suggests the importance of designing the characterisation protocol of an aptamer by considering its final application. Also the aptamer surface density can have a significant influence on the target binding as shown by Simon *et al.* [103]. The authors used a high throughput SPR imaging system (SPRi) and showed that the optimal surface density of the aptamer is dependent on the target size; they did

so by studying two different aptamers, one for the protein immunoglobulin E and one for the peptide 6xHis-tag.

In addition, SPR was directly combined with SELEX to select an aptamer against a structured RNA, derived from the transcription factor *XBP1*. SPR was allowed to follow the evolution of the aptamers throughout the SELEX cycles and the SPR-SELEX combination resulted in a simultaneous aptamer selection and real-time evaluation of their binding affinity [104]. A similar principle was reported by Jia *et al.* [105] in which SPRI was combined with SELEX to select and characterise aptamers against the protein lipocalin-1.

Surface-enhanced Raman scattering

SERS showed a wide applicability in multi-analytical aptamer-target characterisation studies. Since 2009, SERS potential for monitoring aptamer conformational changes was uncovered. Conformational changes can be quantified by a spectral cross-correlation function, as described by Neumann *et al.* [106] making SERS a suitable complementary technique in aptamer binding studies. Recently, Cui *et al.* [107] successfully combined the data obtained via BLI, SERS and molecular dynamics (MD) simulations for determining the affinity and conformational changes of the theophylline-binding aptamer upon binding of its target, as summarised in Figure 6. The authors took advantage from the label-free, sensitive and real-time technology of BLI to first evaluate the affinity, which often proceeds aptasensing design [108,109] but does not allow to gain information about conformational changes. The latter were monitored indirectly by comparison of the differences in the SERS spectra and carefully analysing the signals related to the aptamer nucleobases.

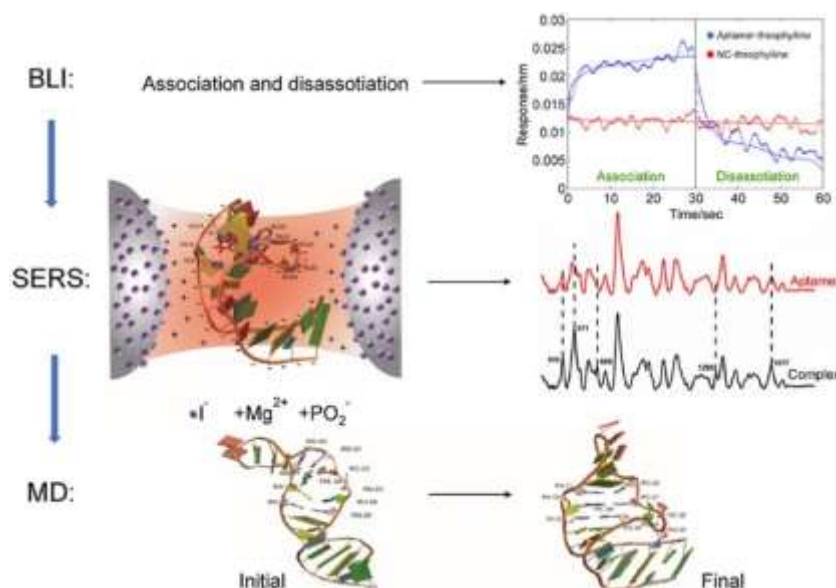


Figure 6 – The kinetic parameters of theophylline binding to its aptamer were determined using BLI. The combination with SERS and MD simulations allowed the authors to analyse the conformational changes of the aptamer and finally an in-depth understanding of the aptamer-theophylline binding process was obtained. Reprinted with permission from [107]. Copyright (2020) Elsevier.

2.2.2 Electrochemical techniques

Highly sensitive electrochemical techniques, such as electrochemical impedance spectroscopy, but also potentiometric and amperometric techniques, are widely applied in aptasensing [110–112]. However, their application is often limited to monitor the aptamer-target binding event and not to evaluate kinetic or affinity parameters. Nevertheless, Eissa *et al.* reported an electrochemical SELEX platform in which the K_D values of aptamers against 11-deoxycortisol, immobilised at the electrode surface, were determined via square wave

voltammetry by estimating peak current changes upon aptamer binding [113]. Afterwards, the authors selected one aptamer and applied this in a voltammetric label-free aptasensor. Despite the sensitivity of electrochemical-based sensing strategies, even down to the sub-picomolar range [114], they cannot be used to gain further information about the binding event itself.

2.2.3 Mass-sensitive techniques

Quartz crystal microbalance (QCM) is a label-free, mass-sensitive technique in which the resonance frequency of a quartz crystal varies when the mass on the crystal surface changes [115]. Challier *et al.* [29] used QCM, with dissipation monitoring, to study the effect of surface immobilisation of a L-tyrosinamide-binding aptamer. The K_D was estimated from these experiments by looking at shifts in QCM frequency at various concentrations of the target and fitting these data. It was found that these values were up to two orders of magnitude higher than the K_D determined in solution by ITC. These results show again that immobilisation of the aptamer can have a significant influence on the aptamer performance. QCM was also combined with SELEX by Hu *et al.* [116] to monitor the QCM frequency in real-time throughout each selection round and consequently determine the K_D -values of selected aptamer candidates for acrylamide.

3. Structural studies

Aptamers can adopt a wide range of structural motifs, such as loops, hairpins, pseudoknots and G-quadruplexes. These structural elements define the aptamer structure, which is crucial for target binding. Understanding the structural properties and conformational changes upon binding of a target is therefore a fundamental step in the understanding of how an aptamer works and further prove its selectivity toward the ligand. In this frame, we describe the main analytical techniques to study aptamer structure which can be divided in two main sections: high-resolution techniques where an atomic resolution is reached (section 3.1) and low-resolution techniques which provide general information on the size and shape of the aptamer (section 3.2).

3.1 High-resolution techniques

There are only three techniques which can provide atomic resolution structures: nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography and electron microscopy (EM). NMR spectroscopy, commonly used for aptamers in complex with small molecules, and X-ray crystallography, mainly used for aptamer-protein complexes, are discussed in more detail in section 3.1.1 and 3.1.2, respectively. Finally, in the category of EM, especially cryo-EM is interesting since molecules are frozen but hydrated at cryogenic temperatures [117]. Therefore, cryo-EM allows imaging of samples in their native state. Despite its great potential, cryo-EM has not been used to determine aptamer structure so far.

3.1.1 NMR spectroscopy

NMR spectroscopy has been used since the 1990s to study the 3D structure of aptamers and aptamer-target complexes. The use of NMR spectroscopy is limited to relatively small biomolecules (< 40 kilodaltons) due to broadening and overlap of NMR signals when molecules become too complex. Secondary and tertiary structure determination of free-form aptamers is difficult since they often have an undefined, heterogeneous form in their unbound state. However, NMR spectroscopy is an excellent tool to study the structures of aptamer in their bound state. Currently, over one hundred aptamer-target structures have been determined by NMR spectroscopy [118].

Structure determination of aptamers is often difficult due to overlap of signals, but even when the structure cannot be determined, important information on aptamers can be obtained. The imino proton region of

guanosine, thymidine and uridine (10 to 15 ppm) provides information about the base pairing in aptamers, as was demonstrated by Minagawa *et al.* [119] and by Philip Johnson's group [32,120,121]. The first used the imino proton signals of an aptamer against salivary α -amylase measured at different temperatures to obtain information on the thermal stability of the aptamer. The disappearance of signals from 40 °C onwards indicated disruption of the base pairs and the consequent unfolding of the aptamer. Neves *et al.* [32] showed that the imino signals can indicate how well-structured certain aptamers are. They did so by using a set of cocaine-binding aptamers with different stem lengths. Aptamers with many sharp peaks in the imino region were found to be rigidly structured while fewer and broad peaks are attributed to loosely structured and dynamic aptamers. Similar conclusions were drawn by analysing the exchange rate of imino protons of two of these cocaine-binding aptamers. The exchange rate values were higher for the more dynamic aptamers compared to the rigidly structured one [120]. Upon binding of the target, the exchange rate was reduced indicating a more stable and less solvent accessible structure. Interestingly, base pairs which were already preformed also experience a reduction in dynamics upon binding [121]. Verdonck *et al.* [122] studied a duplex with imidazole-tethered thymidines. These thymidines can interact with a nucleotide in the opposite strand and form a so-called pK_a-motif, which increases the thermal stability of the duplex [123]. Using a combination of MD simulations and nuclear Overhauser spectroscopy, the authors confirmed the formation of pK_a-motifs in the duplex. Furthermore, they applied the same principle to a L-argininamide-binding aptamer and found the motif occurs and stabilises the aptamer while maintaining the binding capacity.

By comparison of the NMR signals in the absence and presence of a target, conformational changes upon target binding can be monitored. Oguro *et al.* [124] showed this for a spermine-binding aptamer which has a stem-loop structure. The terminal side of the structure is loose while the loop side forms a stable structure, and the binding of spermine induced a conformational change. More recently, structural changes of a tetramethylrhodamine-binding aptamer upon binding of the target were monitored [125]. The authors found the aptamer undergoes extensive structural changes and forms a stable three-way junction upon binding. Slavkovic *et al.* [126] used a combination of thermal stability, ITC and NMR experiments to determine why the ATP-binding aptamer binds two copies of the target cooperatively. By investigating the aptamer and two related constructs, a population shift mechanism was discovered which results in a cooperative binding mechanism.

Besides the commonly used ¹H NMR, also fluorine-19 (¹⁹F) NMR, first reported by Marshall *et al.* [127] in 1977, can be used to analyse structural features of nucleic acids. ¹⁹F NMR has several advantages compared to ¹H NMR [128]: (1) the ¹⁹F nucleus is highly sensitive to changes in the environment and is therefore ideal to study conformational changes; (2) ¹⁹F is absent in natural compounds and incorporation of a ¹⁹F probe in nucleic acids, either nucleotide-based within the sequence [129] or at the end of the sequence [130], generates spectra which are easy to interpret. Ishizuka *et al.* [131] used ¹⁹F NMR to investigate the thrombin-binding aptamer with G-quadruplex structure (Figure 7). Several aptamer variants with a single ¹⁹F probe incorporated at each thymine position were used to: (1) monitor structural changes in function of temperature and (2) determine thermodynamic parameters of the G-quadruplex. Moreover, the binding of thrombin to the aptamer was followed by considering the changes in chemical shift.

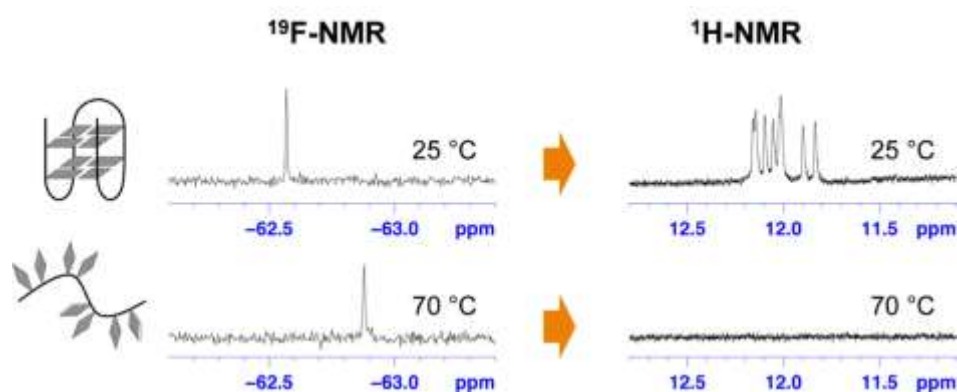


Figure 7 – ^{19}F NMR and ^1H NMR spectra of the thrombin-binding aptamer with an ^{19}F -labelled nucleotide at the T10 position at 25 or 70 °C. The quadruplex is unfolded at 70 °C which results in a change of chemical shift in ^{19}F NMR and a disappearance of the signals in ^1H NMR. Reprinted with permission from [131]. Copyright (2017) American Chemical Society.

NMR was also used to obtain structural information on nucleic acids in living cells [132,133]. Recently, Broft *et al.* [134] studied a riboswitch using in-cell NMR and observed binding of 2'-deoxyguanosine to the aptamer domain of the riboswitch. This technique holds great potential to investigate structure-function relationships of aptamers within cells to monitor aptamer treatment.

3.1.2 X-ray crystallography

X-ray crystallography is mostly used to investigate aptamers in complex with relatively large molecules, such as proteins (a detailed overview of the use of X-ray crystallography to study aptamer-protein interactions was provided by Gelin *et al.* [135]). A crystal structure provides detailed information on the interactions between the two binding partners. It is, however, often difficult to obtain well-diffracting crystals since the co-crystallisation depends on many factors such as the purity of the compounds, the conformation of the aptamer and the ratio between the aptamer and its target. Therefore, finding the conditions which provide suitable diffracting crystals is usually a time-consuming process of trial and error [136]. Dynamic and conformationally flexible molecules are difficult to crystallise as was recently shown by Schmidt *et al.* [137]. The authors studied an aptamer against the flexible peptide ghrelin and were not able to crystallise the aptamer or peptide alone due to the many conformational changes they undergo. The crystal structure of the aptamer-ghrelin complex was, however, determined since this might be more rigid compared to the two components alone.

Regular X-ray crystallography provides static information of a molecule, but Stagno *et al.* [138] used serial femtosecond crystallography (SFX) with an X-ray free-electron laser to determine transient states of the adenine riboswitch aptamer domain. During SFX experiments, snapshots of static structures are recorded and over time this indicates conformational changes of processes in the picosecond to millisecond timescale. Upon binding of adenine, structures of four states of the adenine riboswitch aptamer domain were determined. Later, Ding *et al.* [139] used SFX in combination with NMR to determine the conformational flexibility of the same riboswitch aptamer domain in both the free and bound form. This technique holds great potential to obtain high resolution data on conformational changes of aptamers and their complexes.

3.2 Low-resolution techniques

Several techniques can provide structural information on the aptamer conformation without providing a full atomic resolution structure. These so-called low-resolution techniques provide general information on the size and shape of the aptamer or provide information on secondary structure elements. The two frequently used techniques providing this type of information are circular dichroism (CD) spectroscopy and small-angle X-ray scattering (SAXS).

3.2.1 Circular dichroism spectroscopy

CD spectroscopy is a widely used technique to study the secondary structure of aptamers and their conformational changes upon binding. Various secondary structural elements have distinguished CD spectra with specific maxima and minima in ellipticity in function of the wavelength. The main advantages of CD spectroscopy are that it is relatively inexpensive, fast and has a high sensitivity [140]. As CD spectroscopy only determines the global features of macromolecules, it is often used in combination with other techniques.

CD spectroscopy is frequently applied to study aptamers with a G-quadruplex structure. Zhang *et al.* [141] studied the PW17 aptamer in which G-quadruplex formation is induced by the presence of potassium ions (Figure 8). It was shown that at low K^+ concentrations the aptamer is loose and unstable and upon increasing the K^+ concentration the aptamer folded into a parallel G-quadruplex. Wiedman *et al.* [142] determined a G-quadruplex structure of an aptamer against posaconazole, an antifungal drug. They found that the G-quadruplex structure is essential for binding the target and that Mg^{2+} enhances the folding of the G-quadruplex. CD spectroscopy was also used in combination with online predicting tools for an aptamer against the antibody rituximab [143]. Six different aptamers were studied, and it was found that two aptamers fold into a G-quadruplex, while the other four adopt a structure similar to B-DNA helices. More recently, Aljohani *et al.* [144] used the change in the intensity of the ellipticity as a measure of the conformational change and strength of the binding. They studied an aptamer against dabigatran etexilate, an anti-coagulant, together with a truncated form and found that upon addition of the target the truncated form has a more sensitive response. Therefore, they stated the truncated form is a better binder than the native aptamer.

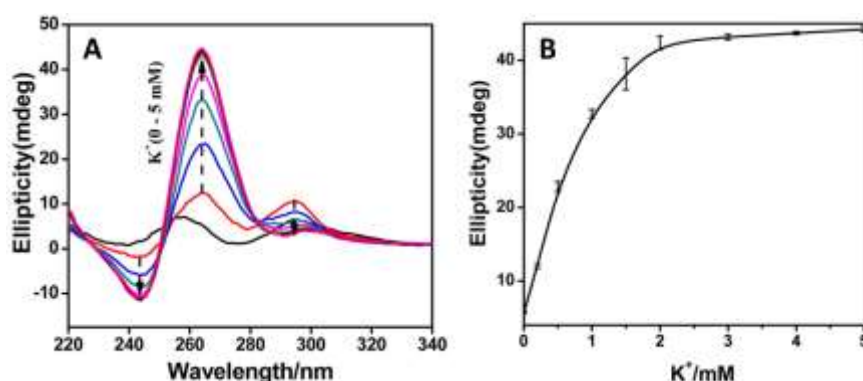


Figure 8 – A) CD spectra of the PW17 aptamer with G-quadruplex structure. Spectra are recorded with K^+ concentrations from 0.02 mM to 5 mM from bottom to top. B) The CD intensity at 264 nm is plotted in function of the K^+ concentration. The CD spectroscopy results show the G-quadruplex formation induced by the presence of K^+ and that from 2 mM K^+ onwards a stable structure is formed. Reprinted with permission from [141]. Copyright (2016) American Chemical Society.

CD spectroscopy can also be used to investigate the structural stability of aptamers. Yang *et al.* [54] reported CD-melting curves of a thrombin-binding aptamer in the free form and bound to the natural product jatrorrhizine. It was found that this target increases the thermal stability, and thus the conformational stability, of the G-quadruplex structure.

3.2.2 Small-angle X-ray scattering

SAXS is often used as a complementary method and allows to investigate the spatial molecule structure and conformational changes induced by either varying conditions (e.g. temperature and pH) or target binding [145]. From SAXS results, a spatial distribution of the electron density of the aptamer can be built to obtain the general aptamer shape (Figure 9).

The structure of two aptamers against the interleukin-6 receptor were investigated using SAXS and were found to have an elongated shape and existed as a dimer in solution [146,147]. Recently, Schmidt *et al.* [137] studied an aptamer against the peptide ghrelin with non-natural L-nucleotides which are resistant to nucleases. SAXS experiments showed the aptamer undergoes a conformational change and becomes more compact upon binding of the target. Moreover, the experiments allowed to derive the molecular weight and suggested the aptamer exists as a dimer in solution, while presenting a 1:1 stoichiometry aptamer-ghrelin. Tomilin *et al.* [148] proposed a four-step process to obtain a 3D structure of a thrombin-binding aptamer. From SAXS results, a structural envelope was built. Using MD simulations, a 3D model was built based on the primary and secondary structure of the aptamer and in a final step this model was compared with the experimental SAXS data to further refine the model.

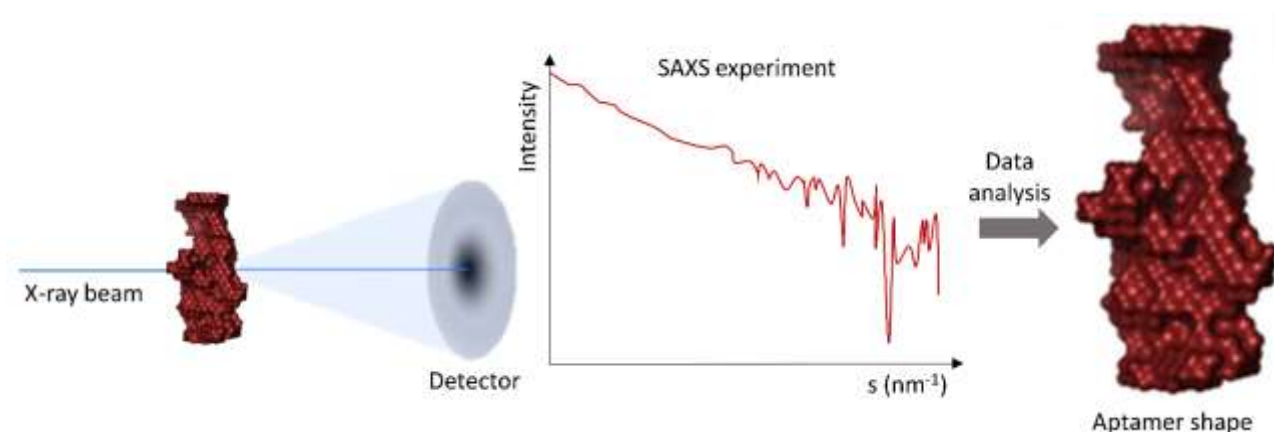


Figure 9 – The general working principle to obtain information on the aptamer shape using SAXS. SAXS experiments are performed on an aptamer in solution and consequently a structural envelope is built from these SAXS results. Adapted from Tomilin *et al.* [148].

4. Conclusions

This review aimed the description of the main analytical tools for aptamer characterisation, including their limits and potential, to answer the need for a more critical evaluation of aptamer performance recently emphasised in the literature. By dividing the analytical techniques presented in respect of the information they provide, we aim to help researchers to identify the most suitable characterisation protocol for the desired application. Therefore, it is fundamental to distinguish kinetic and thermodynamic studies from structural ones. The examples presented per section underline the consequences that the choice of a certain analytical protocol can have and how misleading the data interpretation can be, with further implications in the following applications (*i.e.* non-functioning aptamers applied in aptasensors).

The techniques discussed in this review are compared in Figure 10 concerning the type of information provided. Several techniques provide quantitative or semi-quantitative kinetic and/or thermodynamic information on the binding event (*e.g.* binding affinity and/or stoichiometry), while they do not provide any structural information. Only two techniques, *i.e.* native (IM-)MS and SERS, provide information on the binding event and structure of the aptamer-target complex. Both techniques only provide qualitative information on the binding (*e.g.* ranking of binding affinities) and low-resolution structural information (*e.g.* general shape). Low-resolution structural information can also be obtained by CD and SAXS but these do not provide any information on the binding event. Finally, high-resolution structural information (*e.g.* a structure at atomic resolution) can be obtained by NMR spectroscopy or X-ray crystallography.

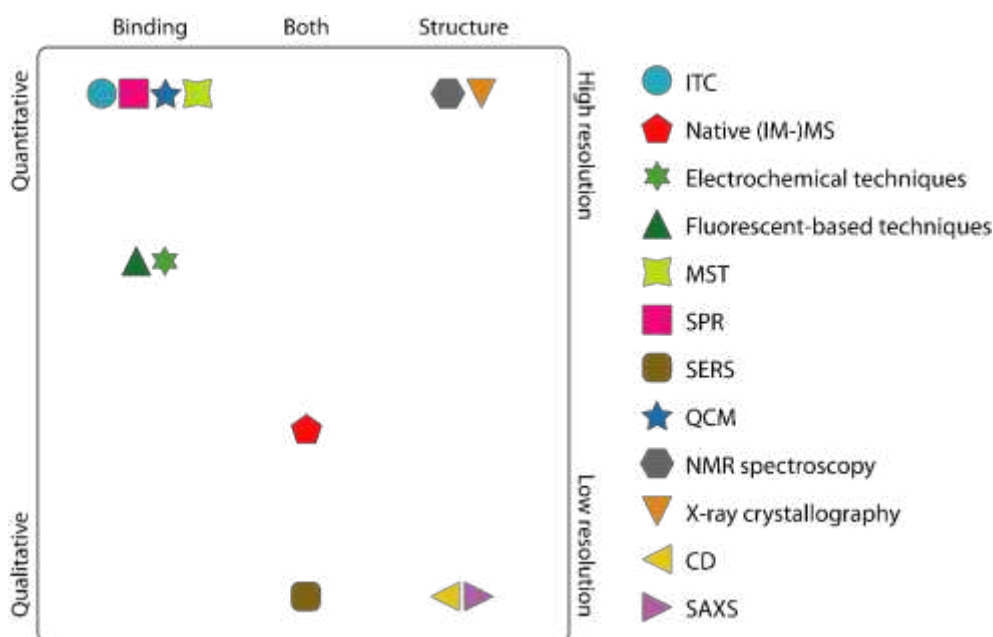


Figure 10 – Overview of the information on aptamer-target complexes obtained using each of the discussed techniques. The information concerns the binding, the structure or both. Binding information can be the quantitative, semi-quantitative or qualitative while for structural information the level of resolution is considered (high or low).

These general observations lead to the conclusion that multi-analytical approaches are recommended to thoroughly characterise aptamers and aptamer-target complexes. In this frame, the design of the analytical protocol plays a key-role and the choice of the analytical tools require an extensive knowledge and continuous updates. The critical choice of the analytical tools needs to consider the final application of the aptamer since certain techniques can report different affinities of a given aptamer. Indeed, the examples throughout the text showed that introducing a label to the aptamer or immobilising the aptamer can alter the aptamer behaviour (*i.e.* testing the aptamer complex in solution or at a confined surface). Therefore, a rational choice between label-free, labelled or immobilised approaches is recommended.

Apart from the accurate evaluation of the applicability and performance of the described analytical techniques, several other parameters should be taken into account when designing the characterization protocol, such as costs, speed, amount of sample and user-friendliness (Figure 11). The costs include the instrument costs, its maintenance and the protocol-related costs (*e.g.* sample preparation). The speed ranges from minutes to hours for an analysis and is important to consider, especially for screening purposes for which you preferably have a fast technique. The amount of sample includes both the concentration (nM to mM-range) and volume (μL-range) required to perform an analysis. Finally, the user-friendliness of the techniques is evaluated. This parameter does not only include the easiness of performing the experiment but also following procedures such as data analysis and interpretation.

	Costs	Speed	Amount of sample	User-friendliness
ITC	€€€	🏃🏃	💧💧💧	🎓🎓🎓
Native (IM-)MS	€€€€€	🏃🏃🏃🏃🏃	💧	🎓🎓🎓🎓🎓
Electrochemical techniques	€	🏃🏃🏃🏃🏃	💧	🎓🎓🎓
Fluorescent-based techniques	€€	🏃🏃🏃	💧	🎓
MST	€€€	🏃🏃🏃🏃	💧	🎓🎓🎓
SPR	€€€€	🏃🏃🏃	💧	🎓🎓🎓
SERS	€€€	🏃🏃🏃🏃🏃	💧💧💧	🎓🎓🎓
QCM	€€€€	🏃🏃🏃🏃	💧	🎓🎓🎓
NMR spectroscopy	€€€€€	🏃🏃🏃	💧💧💧💧	🎓🎓🎓🎓
X-ray crystallography	€€€€€	🏃	💧💧💧	🎓🎓🎓🎓🎓
CD	€€	🏃🏃🏃🏃🏃	💧💧💧	🎓
SAXS	€€€€€	🏃🏃	💧💧💧	🎓🎓🎓🎓🎓

Figure 11 – Comparison of the discussed techniques in terms of costs, speed, amount of sample required and user-friendliness.

It is worth noticing that the rising interest in an in-depth characterisation of aptamer-target complexes is leading to the development of new analytical tools often already known for other applications. One of the examples is kinetic ITC for studying the kinetics of aptamer-target binding or on a structural side, cryo-EM. Despite the variety of tools already available it is fundamental to be able to *map the gaps* of these techniques in terms of information provided and limits of applicability. Certain multi-analytical approaches cannot be afforded/applied in routine studies for the development of aptamers for medical treatments, etc. There is still the urgent need to design, or redesign, new analytical tools able to provide high performance and rapid analysis with affordable costs.

Acknowledgments

This work was supported by the Research Foundation Flanders (FWO) (grant number G054819N and E.D. acknowledges a FWO fellowship with grant number 1S65717N), R.C. acknowledges a MSCA (grant number 842219). Dr Fabio Bottari for revising the manuscript.

Bibliography

- [1] S. Cai, J. Yan, H. Xiong, Y. Liu, D. Peng, Z. Liu, Investigations on the interface of nucleic acid aptamers and binding targets, *Analyst*. 143 (2018) 5317–5338. <https://doi.org/10.1039/c8an01467a>.
- [2] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, *Science*. 249 (1990) 505–510. <https://doi.org/10.1126/science.2200121>.
- [3] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, *Nature*. 346 (1990) 818–822. <https://doi.org/10.1038/346818a0>.
- [4] R. Green, A.D. Ellington, J.W. Szostak, In vitro genetic analysis of the Tetrahymena self-splicing intron, *Nature*. 347 (1990) 406–408. <https://doi.org/10.1038/347406a0>.
- [5] N. Komarova, A. Kuznetsov, Inside the Black Box: What Makes SELEX Better?, *Molecules*. 24 (2019) 3598. <https://doi.org/10.3390/molecules24193598>.
- [6] S.M. Nimjee, R.R. White, R.C. Becker, B.A. Sullenger, Aptamers as Therapeutics, *Annu. Rev. Pharmacol. Toxicol.* 57 (2017) 61–79. <https://doi.org/10.1146/annurev-pharmtox-010716-104558>.
- [7] Z. Fu, J. Xiang, Aptamers, the nucleic acid antibodies, in cancer therapy, *Int. J. Mol. Sci.* 21 (2020) 2793. <https://doi.org/10.3390/ijms21082793>.
- [8] A. Chen, S. Yang, Replacing antibodies with aptamers in lateral flow immunoassay, *Biosens. Bioelectron.* 71 (2015) 230–242. <https://doi.org/10.1016/j.bios.2015.04.041>.
- [9] D. Wang, Y. Peng, Z. Deng, Y. Tan, Y. Su, H. Kuai, L. Ai, Z. Huang, X. Wang, X. Zhang, W. Tan, Modularly Engineered Solid-Phase Synthesis of Aptamer-Functionalized Small Molecule Drugs for Targeted Cancer Therapy, *Adv. Ther.* 3 (2020) 2000074. <https://doi.org/10.1002/adtp.202000074>.
- [10] J. Jeon, J. Lee, J. in So, J.B. Lee, H. Lee, Y. Chang, S. Shin, J. Jo, C. Ban, Homogeneous fluorescent aptasensor for active tuberculosis diagnosis by direct quantification of circulating TB7.7 based on aptamer beacon with graphene oxide, *Sensors Actuators, B Chem.* 317 (2020) 128126. <https://doi.org/10.1016/j.snb.2020.128126>.
- [11] S. Chakraborty, Z.Y. Dlie, S. Chakraborty, S. Roy, B. Mukherjee, S.E. Besra, S. Dewanjee, A. Mukherjee, P.K. Ojha, V. Kumar, R. Sen, Aptamer-Functionalized Drug Nanocarrier Improves Hepatocellular Carcinoma toward Normal by Targeting Neoplastic Hepatocytes, *Mol. Ther. - Nucleic Acids*. 20 (2020) 34–49. <https://doi.org/10.1016/j.omtn.2020.01.034>.
- [12] S. Catuogno, M.T. Di Martino, S. Nuzzo, C.L. Esposito, P. Tassone, V. de Franciscis, An Anti-BCMA RNA Aptamer for miRNA Intracellular Delivery, *Mol. Ther. - Nucleic Acids*. 18 (2019) 981–990. <https://doi.org/10.1016/j.omtn.2019.10.021>.
- [13] Y. Jiang, M. Shi, Y. Liu, S. Wan, C. Cui, L. Zhang, W. Tan, Aptamer/AuNP Biosensor for Colorimetric Profiling of Exosomal Proteins, *Angew. Chemie - Int. Ed.* 56 (2017) 11916–11920. <https://doi.org/10.1002/anie.201703807>.
- [14] Y. Jia, G. Zhou, X. Wang, Y. Zhang, Z. Li, P. Liu, B. Yu, J. Zhang, A metal-organic framework/aptamer system as a fluorescent biosensor for determination of aflatoxin B1 in food samples, *Talanta*. 219 (2020) 121342. <https://doi.org/10.1016/j.talanta.2020.121342>.
- [15] M. Ilgu, M. Nilsen-Hamilton, Aptamers in analytics, *Analyst*. 141 (2016) 1551–1558. <https://doi.org/10.1039/c5an01824b>.
- [16] R. Stoltenburg, C. Reinemann, B. Strehlitz, FluMag-SELEX as an advantageous method for DNA aptamer selection, *Anal. Bioanal. Chem.* 383 (2005) 83–91. <https://doi.org/10.1007/s00216-005-3388-9>.
- [17] R. Stoltenburg, N. Nikolaus, B. Strehlitz, Capture-SELEX: Selection of DNA aptamers for aminoglycoside antibiotics, *J. Anal. Methods Chem.* 1 (2012). <https://doi.org/10.1155/2012/415697>.
- [18] J.W. Park, R. Tatavarty, D.W. Kim, H.T. Jung, M.B. Gu, Immobilization-free screening of aptamers assisted by graphene oxide, *Chem. Commun.* 48 (2012) 2071–2073. <https://doi.org/10.1039/c2cc16473f>.
- [19] C. Zong, J. Liu, The arsenic-binding aptamer cannot bind arsenic: critical evaluation of aptamer selection and binding, *Anal. Chem.* 91 (2019) 10887–10893. <https://doi.org/10.1021/acs.analchem.9b02789>.
- [20] F. Bottari, E. Daems, A.M. de Vries, P. Van Wielendaele, S. Trashin, R. Blust, F. Sobott, A. Madder, J.C.

- Martins, K. De Wael, Do Aptamers Always Bind? The Need for a Multifaceted Analytical Approach When Demonstrating Binding Affinity between Aptamer and Low Molecular Weight Compounds, *J. Am. Chem. Soc.* 142 (2020) 19622–19630. <https://doi.org/10.1021/jacs.0c08691>.
- [21] X. Tao, F. He, X. Liu, F. Zhang, X. Wang, Y. Peng, J. Liu, Detection of chloramphenicol with an aptamer-based colorimetric assay: critical evaluation of specific and unspecific binding of analyte molecules, *Microchim. Acta.* 187 (2020) 668. <https://doi.org/10.1007/s00604-020-04644-6>.
- [22] M. McKeague, A. De Girolamo, S. Valenzano, M. Pascale, A. Ruscito, R. Velu, N.R. Frost, K. Hill, M. Smith, E.M. McConnell, M.C. DeRosa, Comprehensive Analytical Comparison of Strategies Used for Small Molecule Aptamer Evaluation, *Anal. Chem.* 87 (2015) 8608–8612. <https://doi.org/10.1021/acs.analchem.5b02102>.
- [23] R. Miranda-Castro, N. de-los-Santos-Ávarez, M.J. Lobo-Castañón, Characterization of Aptamer-Ligand Complexes, in: Y. Dong (Ed.), *Aptamers Anal. Appl. Affin. Acquis. Method Des.*, 2018: pp. 127–172.
- [24] Y. Qin, X. Yang, J. Zhang, X. Cao, Target capturing performance of microfluidic channel surface immobilized aptamers: the effects of spacer lengths, *Biomed. Microdevices.* 21 (2019) 54. <https://doi.org/10.1007/s10544-019-0403-z>.
- [25] S. Wang, Y. Dong, X. Liang, Development of a SPR aptasensor containing oriented aptamer for direct capture and detection of tetracycline in multiple honey samples, *Biosens. Bioelectron.* 109 (2018) 1–7. <https://doi.org/10.1016/j.bios.2018.02.051>.
- [26] K.A. Yang, H. Chun, Y. Zhang, S. Pecic, N. Nakatsuka, A.M. Andrews, T.S. Worgall, M.N. Stojanovic, High-Affinity Nucleic-Acid-Based Receptors for Steroids, *ACS Chem. Biol.* 12 (2017) 3103–3112. <https://doi.org/10.1021/acscchembio.7b00634>.
- [27] T. Wiseman, S. Williston, J.F. Brandts, L.N. Lin, Rapid measurement of binding constants and heats of binding using a new titration calorimeter, *Anal. Biochem.* 179 (1989) 131–137. [https://doi.org/10.1016/0003-2697\(89\)90213-3](https://doi.org/10.1016/0003-2697(89)90213-3).
- [28] E. Freire, O.L. Mayorga, M. Straume, Isothermal Titration, *Anal. Chem.* 62 (1990) 950–959. <https://doi.org/10.1021/ac00217a715>.
- [29] L. Challier, R. Miranda-Castro, B. Barbe, C. Fave, B. Limoges, E. Peyrin, C. Ravelet, E. Fiore, P. Labbé, L. Coche-Guérente, E. Ennifar, G. Bec, P. Dumas, F. Mavré, V. Noë, Multianalytical study of the binding between a small chiral molecule and a DNA aptamer: Evidence for asymmetric steric effect upon 3'-versus 5'-end sequence modification, *Anal. Chem.* 88 (2016) 11963–11971. <https://doi.org/10.1021/acs.analchem.6b04046>.
- [30] C. Bayraç, H.A. Öktem, Evaluation of *Staphylococcus aureus* DNA aptamer by enzyme-linked aptamer assay and isothermal titration calorimetry, *J. Mol. Recognit.* 30 (2017) 1–8. <https://doi.org/10.1002/jmr.2583>.
- [31] S. Slavkovic, M. Altunisik, O. Reinstein, P.E. Johnson, Structure-affinity relationship of the cocaine-binding aptamer with quinine derivatives, *Bioorganic Med. Chem.* 23 (2015) 2593–2597. <https://doi.org/10.1016/j.bmc.2015.02.052>.
- [32] M.A.D. Neves, A.A. Shoara, O. Reinstein, O. Abbasi Borhani, T.R. Martin, P.E. Johnson, Optimizing Stem Length to Improve Ligand Selectivity in a Structure-Switching Cocaine-Binding Aptamer, *ACS Sensors.* 2 (2017) 1539–1545. <https://doi.org/10.1021/acssensors.7b00619>.
- [33] A.A. Shoara, O. Reinstein, O.A. Borhani, T.R. Martin, S. Slavkovic, Z.R. Churcher, P.E. Johnson, Development of a thermal-stable structure-switching cocaine-binding aptamer, *Biochimie.* 145 (2018) 137–144. <https://doi.org/10.1016/j.biochi.2017.08.010>.
- [34] S. Slavkovic, S.R. Eisen, P.E. Johnson, Designed Alteration of Binding Affinity in Structure-Switching Aptamers through the Use of Dangling Nucleotides, *Biochemistry.* 59 (2020) 663–670. <https://doi.org/10.1021/acs.biochem.9b00630>.
- [35] T. Amato, A. Virgilio, L. Pirone, V. Vellecco, M. Bucci, E. Pedone, V. Esposito, A. Galeone, Investigating the properties of TBA variants with twin thrombin binding domains, *Sci. Rep.* 9 (2019) 1–8. <https://doi.org/10.1038/s41598-019-45526-z>.
- [36] E. Zavyalova, A. Golovin, G. Pavlova, A. Kopylov, Module-Activity Relationship of G-quadruplex Based DNA Aptamers for Human Thrombin, *Curr. Med. Chem.* 20 (2013) 4836.

- [37] O.M. Antipova, G.M. Solius, D.Y. Panteleev, R. V. Reshetnikov, Interaction between thrombin and oligonucleotide RA36 is a two-stage process, *Biochem. Biophys. Res. Commun.* 522 (2020) 1037–1040. <https://doi.org/10.1016/j.bbrc.2019.11.190>.
- [38] S.A. Kantonen, N.M. Henriksen, M.K. Gilson, Evaluation and Minimization of Uncertainty in ITC Binding Measurements: Heat Error, Concentration Error, Saturation, and Stoichiometry, *Biochim. Biophys. Acta - Gen. Subj.* 1861 (2017) 485–498. <https://doi.org/10.1016/j.bbagen.2016.09.002>.
- [39] W.B. Turnbull, A.H. Daranas, On the Value of c : Can Low Affinity Systems Be Studied by Isothermal Titration Calorimetry?, *J. Am. Chem. Soc.* 125 (2003) 14859–14866. <https://doi.org/10.1021/ja036166s> CCC:
- [40] J. Tellinghuisen, Isothermal titration calorimetry at very low c , *Anal. Biochem.* 373 (2008) 395–397. <https://doi.org/10.1016/j.ab.2007.08.039>.
- [41] R. Amano, K. Takada, Y. Tanaka, Y. Nakamura, G. Kawai, T. Kozu, T. Sakamoto, Kinetic and Thermodynamic Analyses of Interaction between a High-Affinity RNA Aptamer and Its Target Protein, *Biochemistry.* 55 (2016) 6221–6229. <https://doi.org/10.1021/acs.biochem.6b00748>.
- [42] M.A.D. Neves, A.A. Shoara, O. Reinstein, O. Abbasi Borhani, T.R. Martin, P.E. Johnson, Optimizing Stem Length to Improve Ligand Selectivity in a Structure-Switching Cocaine-Binding Aptamer, *ACS Sensors.* 2 (2017) 1539–1545. <https://doi.org/10.1021/acssensors.7b00619>.
- [43] Z. Zhang, O. Oni, J. Liu, New insights into a classic aptamer: Binding sites, cooperativity and more sensitive adenosine detection, *Nucleic Acids Res.* 45 (2017) 7593–7601. <https://doi.org/10.1093/nar/gkx517>.
- [44] Y. Li, B. Liu, Z. Huang, J. Liu, Engineering base-excised aptamers for highly specific recognition of adenosine, *Chem. Sci.* 11 (2020) 2735–2743. <https://doi.org/10.1039/d0sc00086h>.
- [45] B.W. Sigurskjold, Exact analysis of competition ligand binding by displacement isothermal titration calorimetry, *Anal. Biochem.* 277 (2000) 260–266. <https://doi.org/10.1006/abio.1999.4402>.
- [46] T.C. Kuo, C.W. Tsai, P.C. Lee, W.Y. Chen, Revisiting the streptavidin-biotin binding by using an aptamer and displacement isothermal calorimetry titration, *J. Mol. Recognit.* 28 (2015) 125–128. <https://doi.org/10.1002/jmr.2366>.
- [47] D. Burnouf, E. Ennifar, S. Guedich, B. Puffer, G. Hoffmann, G. Bec, F. Disdier, M. Baltzinger, P. Dumas, KinITC: A new method for obtaining joint thermodynamic and kinetic data by isothermal titration calorimetry, *J. Am. Chem. Soc.* 134 (2012) 559–565. <https://doi.org/10.1021/ja209057d>.
- [48] J.M. Di Trani, N. Moitessier, A.K. Mittermaier, Measuring Rapid Time-Scale Reaction Kinetics Using Isothermal Titration Calorimetry, *Anal. Chem.* 89 (2017) 7022–7030. <https://doi.org/10.1021/acs.analchem.7b00693>.
- [49] Á. Piñeiro, E. Muñoz, J. Sabín, M. Costas, M. Bastos, A. Velázquez-Campoy, P.F. Garrido, P. Dumas, E. Ennifar, L. García-Río, J. Rial, D. Pérez, P. Fraga, A. Rodríguez, C. Coteló, AFFINImeter: A software to analyze molecular recognition processes from experimental data, *Anal. Biochem.* 577 (2019) 117–134. <https://doi.org/10.1016/j.ab.2019.02.031>.
- [50] E. Munoz, J. Sabin, J. Rial, D. Perez, E. Ennifar, P. Dumas, A. Pineiro, Thermodynamic and Kinetic Analysis of Isothermal Titration Calorimetry Experiments by Using KinITC in AFFINImeter, in: *Microcalorim. Biol. Mol.*, 2019: pp. 225–239. <https://doi.org/10.7551/mitpress/8119.003.0022>.
- [51] J.M. Di Trani, S. De Cesco, R. O’Leary, J. Plescia, C.J. Do Nascimento, N. Moitessier, A.K. Mittermaier, Rapid measurement of inhibitor binding kinetics by isothermal titration calorimetry, *Nat. Commun.* 9 (2018). <https://doi.org/10.1038/s41467-018-03263-3>.
- [52] F. Sobott, M.G. Mccammon, H. Hernández, C. V. Robinson, M. Karplus, C.M. Dobson, G. Scoles, A.H. Zewail, P.F. McMillan, J.L. Finney, The flight of macromolecular complexes in a mass spectrometer, *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* 363 (2005) 379–391. <https://doi.org/10.1098/rsta.2004.1498>.
- [53] E.B. Erba, C. Petosa, The emerging role of native mass spectrometry in characterizing the structure and dynamics of macromolecular complexes, *Protein Sci.* 24 (2015) 1176–1192. <https://doi.org/10.1002/pro.2661>.
- [54] P. Yang, X. Wang, Z. Gu, H. Li, D.D.Y. Chen, X. Yang, Evaluation of the binding of natural products with thrombin binding aptamer G-quadruplex using electrospray ionization mass spectrometry and

spectroscopic methods, *Talanta*. 200 (2019) 424–431.
<https://doi.org/10.1016/j.talanta.2019.03.080>.

- [55] A. Marchand, F. Rosu, R. Zenobi, V. Gabelica, Thermal Denaturation of DNA G-Quadruplexes and Their Complexes with Ligands: Thermodynamic Analysis of the Multiple States Revealed by Mass Spectrometry, *J. Am. Chem. Soc.* 140 (2018) 12553–12565. <https://doi.org/10.1021/jacs.8b07302>.
- [56] B. Gülbakan, K. Barylyuk, P. Schneider, M. Pillong, G. Schneider, R. Zenobi, Native Electrospray Ionization Mass Spectrometry Reveals Multiple Facets of Aptamer-Ligand Interactions: From Mechanism to Binding Constants, *J. Am. Chem. Soc.* 140 (2018) 7486–7497.
<https://doi.org/10.1021/jacs.7b13044>.
- [57] M. Porrini, F. Rosu, C. Rabin, L. Darré, H. Gómez, M. Orozco, V. Gabelica, Compaction of Duplex Nucleic Acids upon Native Electrospray Mass Spectrometry, *ACS Cent. Sci.* 3 (2017) 454–461.
<https://doi.org/10.1021/acscentsci.7b00084>.
- [58] E. Daems, D. Dewaele, K. Barylyuk, K. De Wael, F. Sobott, Aptamer-ligand recognition studied by native ion mobility-mass spectrometry, *Talanta*. 224 (2021) 121917.
<https://doi.org/10.1016/j.talanta.2020.121917>.
- [59] O. Reinstein, M. Yoo, C. Han, T. Palmo, S.A. Beckham, M.C.J. Wilce, P.E. Johnson, Quinine binding by the cocaine-binding aptamer. thermodynamic and hydrodynamic analysis of high-affinity binding of an off-target ligand, *Biochemistry*. 52 (2013) 8652–8662. <https://doi.org/10.1021/bi4010039>.
- [60] M.N. Stojanovic, P. De Prada, D.W. Landry, Aptamer-based folding fluorescent sensor for cocaine, *J. Am. Chem. Soc.* 123 (2001) 4928–4931. <https://doi.org/10.1021/ja0038171>.
- [61] D. Roncancio, H. Yu, X. Xu, S. Wu, R. Liu, J. Debord, X. Lou, Y. Xiao, A label-free aptamer-fluorophore assembly for rapid and specific detection of cocaine in biofluids, *Anal. Chem.* 86 (2014) 11100–11106. <https://doi.org/10.1021/ac503360n>.
- [62] L. Challier, R. Miranda-Castro, D. Marchal, V. Noël, F. Mavré, B. Limoges, Kinetic rotating droplet electrochemistry: A simple and versatile method for reaction progress kinetic analysis in microliter volumes, *J. Am. Chem. Soc.* 135 (2013) 14215–14228. <https://doi.org/10.1021/ja405415q>.
- [63] H. Guyon, F. Mavré, M. Catala, S. Turcaud, F. Brachet, B. Limoges, C. Tisné, L. Micouin, Use of a redox probe for an electrochemical RNA-ligand binding assay in microliter droplets, *Chem. Commun.* 53 (2017) 1140–1143. <https://doi.org/10.1039/c6cc07785d>.
- [64] S. Xie, Y. Chai, Y. Yuan, L. Bai, R. Yuan, Development of an electrochemical method for Ochratoxin A detection based on aptamer and loop-mediated isothermal amplification, *Biosens. Bioelectron.* 55 (2014) 324–329. <https://doi.org/10.1016/j.bios.2013.11.009>.
- [65] H. Cao, X. Fang, H. Li, H. Li, J. Kong, Ultrasensitive detection of mucin 1 biomarker by immuno-loop-mediated isothermal amplification, *Talanta*. 164 (2017) 588–592.
<https://doi.org/10.1016/j.talanta.2016.07.018>.
- [66] A. Martin, L. Bouffier, K.B. Grant, B. Limoges, D. Marchal, Real-time electrochemical LAMP: A rational comparative study of different DNA intercalating and non-intercalating redox probes, *Analyst*. 141 (2016) 4196–4203. <https://doi.org/10.1039/c6an00867d>.
- [67] J. Jaeger, F. Groher, J. Stamm, D. Spiehl, J. Braun, E. Dörsam, B. Suess, Characterization and Inkjet Printing of an RNA Aptamer for Paper-Based Biosensing of Ciprofloxacin, *Biosens.* . 9 (2019).
<https://doi.org/10.3390/bios9010007>.
- [68] K.L. Fadock, R.A. Manderville, DNA Aptamer-Target Binding Motif Revealed Using a Fluorescent Guanine Probe: Implications for Food Toxin Detection, *ACS Omega*. 2 (2017) 4955–4963.
<https://doi.org/10.1021/acsomega.7b00782>.
- [69] T. Heyduk, Y. Ma, H. Tang, R.H.B.T.-M. in E. Ebright, Fluorescence anisotropy: Rapid, quantitative assay for protein-DNA and protein-protein interaction, in: *RNA Polym. Assoc. Factors, Part B*, Academic Press, 1996: pp. 492–503. [https://doi.org/https://doi.org/10.1016/S0076-6879\(96\)74039-9](https://doi.org/https://doi.org/10.1016/S0076-6879(96)74039-9).
- [70] D. Zhang, M. Lu, H. Wang, Fluorescence Anisotropy Analysis for Mapping Aptamer-Protein Interaction at the Single Nucleotide Level, *J. Am. Chem. Soc.* 133 (2011) 9188–9191.
- [71] Q. Zhao, Y. Bai, H. Wang, Directing a rational design of aptamer-based fluorescence anisotropy assay for sensitive detection of immunoglobulin E by site-specific binding study, *Talanta*. 217 (2020)

121018. <https://doi.org/https://doi.org/10.1016/j.talanta.2020.121018>.
- [72] L. Cui, Y. Zou, N. Lin, Z. Zhu, G. Jenkins, C.J. Yang, Mass amplifying probe for sensitive fluorescence anisotropy detection of small molecules in complex biological samples, *Anal. Chem.* 84 (2012) 5535–5541. <https://doi.org/10.1021/ac300182w>.
- [73] Q. Zhao, J. Tao, J.S. Uppal, H. Peng, H. Wang, X.C. Le, Nucleic acid aptamers improving fluorescence anisotropy and fluorescence polarization assays for small molecules, *TrAC Trends Anal. Chem.* 110 (2019) 401–409. <https://doi.org/https://doi.org/10.1016/j.trac.2018.11.018>.
- [74] H. Ye, Q. Lu, N. Duan, Z. Wang, GO-amplified fluorescence polarization assay for high-sensitivity detection of aflatoxin B1 with low dosage aptamer probe, *Anal. Bioanal. Chem.* 411 (2019) 1107–1115. <https://doi.org/10.1007/s00216-018-1540-6>.
- [75] L. Sun, Q. Zhao, Direct fluorescence anisotropy approach for aflatoxin B1 detection and affinity binding study by using single tetramethylrhodamine labeled aptamer, *Talanta.* 189 (2018) 442–450. <https://doi.org/10.1016/j.talanta.2018.07.036>.
- [76] R.J. Trachman, R. Cojocar, D. Wu, G. Piszczek, M. Ryckelynck, P.J. Unrau, A.R. Ferré-D'Amaré, Structure-Guided Engineering of the Homodimeric Mango-IV Fluorescence Turn-on Aptamer Yields an RNA FRET Pair, *Structure.* 28 (2020) 776–785.e3. <https://doi.org/https://doi.org/10.1016/j.str.2020.04.007>.
- [77] S. Amaya-González, L. López-López, R. Miranda-Castro, N. de-los-Santos-Álvarez, A.J. Miranda-Ordieres, M.J. Lobo-Castañón, Affinity of aptamers binding 33-mer gliadin peptide and gluten proteins: Influence of immobilization and labeling tags, *Anal. Chim. Acta.* 873 (2015) 63–70. <https://doi.org/https://doi.org/10.1016/j.aca.2015.02.053>.
- [78] D. Breitsprecher, N. Schlinck, D. Witte, S. Duhr, P. Baaske, T. Schubert, Aptamer Binding Studies Using MicroScale Thermophoresis BT - Nucleic Acid Aptamers: Selection, Characterization, and Application, in: G. Mayer (Ed.), Springer New York, New York, NY, 2016: pp. 99–111. https://doi.org/10.1007/978-1-4939-3197-2_8.
- [79] Y. Biniuri, B. Albada, I. Willner, Probing ATP/ATP-Aptamer or ATP-Aptamer Mutant Complexes by Microscale Thermophoresis and Molecular Dynamics Simulations: Discovery of an ATP-Aptamer Sequence of Superior Binding Properties, *J. Phys. Chem. B.* 122 (2018) 9102–9109. <https://doi.org/10.1021/acs.jpcc.8b06802>.
- [80] Y. Yang, Y. Yin, S. Wang, Y. Dong, Simultaneous determination of zearalenone and ochratoxin A based on microscale thermophoresis assay with a bifunctional aptamer, *Anal. Chim. Acta.* 1155 (2021) 338345. <https://doi.org/https://doi.org/10.1016/j.aca.2021.338345>.
- [81] S. Sass, W.F.M. Stöcklein, A. Klevesath, J. Hurpin, M. Menger, C. Hille, Binding affinity data of DNA aptamers for therapeutic anthracyclines from microscale thermophoresis and surface plasmon resonance spectroscopy, *Analyst.* 144 (2019) 6064–6073. <https://doi.org/10.1039/c9an01247h>.
- [82] M. Jauset Rubio, M. Svobodová, T. Mairal, T. Schubert, S. Künne, G. Mayer, C.K. O'Sullivan, β -Conglutin dual aptamers binding distinct aptatopes, *Anal. Bioanal. Chem.* 408 (2016) 875–884. <https://doi.org/10.1007/s00216-015-9179-z>.
- [83] V. Skouridou, T. Schubert, A.S. Bashammakh, M.S. El-Shahawi, A.O. Alyoubi, C.K. O'Sullivan, Aptatope mapping of the binding site of a progesterone aptamer on the steroid ring structure, *Anal. Biochem.* 531 (2017) 8–11. <https://doi.org/https://doi.org/10.1016/j.ab.2017.05.010>.
- [84] M. Prante, T. Schöling, B. Roth, K. Bremer, J. Walter, Characterization of an Aptamer Directed against 25-Hydroxyvitamin D for the Development of a Competitive Aptamer-Based Assay, *Biosens. .* 9 (2019). <https://doi.org/10.3390/bios9040134>.
- [85] R.A. Potyrailo, R.C. Conrad, A.D. Ellington, G.M. Hieftje, Adapting Selected Nucleic Acid Ligands (Aptamers) to Biosensors, *Anal. Chem.* 70 (1998) 3419–3425. <https://doi.org/10.1021/ac9802325>.
- [86] H. Chen, S.-G. Park, N. Choi, J.-I. Moon, H. Dang, A. Das, S. Lee, D.-G. Kim, L. Chen, J. Choo, SERS imaging-based aptasensor for ultrasensitive and reproducible detection of influenza virus A, *Biosens. Bioelectron.* 167 (2020) 112496. <https://doi.org/https://doi.org/10.1016/j.bios.2020.112496>.
- [87] S. Eissa, S. Alkhalidi, R. Chinnappan, A. Siddiqua, M. Abduljabbar, A.M. Abdel Rahman, M. Dasouki, M. Zourob, Selection, characterization, and electrochemical biosensing application of DNA aptamers for sepiapterin, *Talanta.* 216 (2020) 120951. <https://doi.org/10.1016/j.talanta.2020.120951>.

- [88] G. Bayramoglu, V.C. Ozalp, M. Oztekin, M.Y. Arica, Rapid and label-free detection of *Brucella melitensis* in milk and milk products using an aptasensor, *Talanta*. 200 (2019) 263–271. <https://doi.org/10.1016/j.talanta.2019.03.048>.
- [89] M. Jarczewska, Ł. Górski, E. Malinowska, Electrochemical aptamer-based biosensors as potential tools for clinical diagnostics, *Anal. Methods*. 8 (2016) 3861–3877. <https://doi.org/10.1039/c6ay00499g>.
- [90] M.N. Nan, Y. Bi, H.L. Xue, H.T. Long, S.L. Xue, L.M. Pu, D. Prusky, Modification performance and electrochemical characteristics of different groups of modified aptamers applied for label-free electrochemical impedimetric sensors, *Food Chem.* 337 (2021) 127761. <https://doi.org/10.1016/j.foodchem.2020.127761>.
- [91] E. Farjami, R. Campos, J.S. Nielsen, K. V. Gothelf, J. Kjems, E.E. Ferapontova, RNA aptamer-based electrochemical biosensor for selective and label-free analysis of dopamine, *Anal. Chem.* 85 (2013) 121–128. <https://doi.org/10.1021/ac302134s>.
- [92] S. Balamurugan, A. Obubuafo, R.L. McCarley, S.A. Soper, D.A. Spivak, Effect of linker structure on surface density of aptamer monolayers and their corresponding protein binding efficiency, *Anal. Chem.* 80 (2008) 9630–9634. <https://doi.org/10.1021/ac8009559>.
- [93] E.-H. Lee, S.K. Lee, M.J. Kim, S.-W. Lee, Simple and rapid detection of bisphenol A using a gold nanoparticle-based colorimetric aptasensor, *Food Chem.* 287 (2019) 205–213. <https://doi.org/https://doi.org/10.1016/j.foodchem.2019.02.079>.
- [94] M. Shorie, V. Kumar, H. Kaur, K. Singh, V.K. Tomer, P. Sabherwal, Plasmonic DNA hotspots made from tungsten disulfide nanosheets and gold nanoparticles for ultrasensitive aptamer-based SERS detection of myoglobin, *Microchim. Acta*. 185 (2018) 158. <https://doi.org/10.1007/s00604-018-2705-x>.
- [95] L. Sun, L. Wu, Q. Zhao, Aptamer based surface plasmon resonance sensor for aflatoxin B1, *Microchim. Acta*. 184 (2017) 2605–2610. <https://doi.org/10.1007/s00604-017-2265-5>.
- [96] H. Qian, Y. Huang, X. Duan, X. Wei, Y. Fan, D. Gan, S. Yue, W. Cheng, T. Chen, Fiber optic surface plasmon resonance biosensor for detection of PDGF-BB in serum based on self-assembled aptamer and antifouling peptide monolayer, *Biosens. Bioelectron.* 140 (2019) 111350. <https://doi.org/https://doi.org/10.1016/j.bios.2019.111350>.
- [97] S. Gao, X. Zheng, J. Wu, A biolayer interferometry-based competitive biosensor for rapid and sensitive detection of saxitoxin, *Sensors Actuators, B Chem.* 246 (2017) 169–174. <https://doi.org/10.1016/j.snb.2017.02.078>.
- [98] D.A. Donkor, V. Bhakta, L.J. Eltringham-Smith, A.R. Stafford, J.I. Weitz, W.P. Sheffield, Selection and characterization of a DNA aptamer inhibiting coagulation factor Xla, *Sci. Rep.* 7 (2017) 1–13. <https://doi.org/10.1038/s41598-017-02055-x>.
- [99] R. Karlsson, SPR for molecular interaction analysis: A review of emerging application areas, *J. Mol. Recognit.* 17 (2004) 151–161. <https://doi.org/10.1002/jmr.660>.
- [100] A.L. Chang, M. McKeague, J.C. Liang, C.D. Smolke, Kinetic and Equilibrium Binding Characterization of Aptamers to Small Molecules using a Label-Free, Sensitive, and Scalable Platform, *Anal. Chem.* 86 (2014) 3273–3278. <https://doi.org/10.1021/ac5001527>.
- [101] R.B.M. Schasfoort, Chapter 1. Introduction to Surface Plasmon Resonance, *Handb. Surf. Plasmon Reson.* (2017) 1–26. <https://doi.org/10.1039/9781788010283-00001>.
- [102] H. Minagawa, K. Onodera, H. Fujita, T. Sakamoto, J. Akitomi, N. Kaneko, I. Shiratori, M. Kuwahara, K. Horii, I. Waga, Selection, Characterization and Application of Artificial DNA Aptamer Containing Appended Bases with Sub-nanomolar Affinity for a Salivary Biomarker, *Sci. Rep.* 7 (2017) 1–9. <https://doi.org/10.1038/srep42716>.
- [103] L. Simon, Z. Bognár, R.E. Gyurcsányi, Finding the Optimal Surface Density of Aptamer Monolayers by SPR Imaging Detection-based Aptamer Microarrays, *Electroanalysis*. 32 (2020) 851–858. <https://doi.org/10.1002/elan.201900736>.
- [104] E. Dausse, A. Barré, A. Aimé, A. Groppi, A. Rico, C. Ainali, G. Salgado, W. Palau, E. Daguerre, M. Nikolski, J.J. Toulmé, C. Di Primo, Aptamer selection by direct microfluidic recovery and surface plasmon resonance evaluation, *Biosens. Bioelectron.* 80 (2016) 418–425.

<https://doi.org/10.1016/j.bios.2016.02.003>.

- [105] W. Jia, Z. Lu, H. Yang, H. Li, D. Xu, Elimination terminal fixed region screening and high-throughput kinetic determination of aptamer for lipocalin-1 by surface plasmon resonance imaging, *Anal. Chim. Acta.* 1043 (2018) 158–166. <https://doi.org/10.1016/j.aca.2018.09.018>.
- [106] O. Neumann, D. Zhang, F. Tam, S. Lal, P. Wittung-Stafshede, N.J. Halas, Direct Optical Detection of Aptamer Conformational Changes Induced by Target Molecules, *Anal. Chem.* 81 (2009) 10002–10006. <https://doi.org/10.1021/ac901849k>.
- [107] X. Cui, M. Song, Y. Liu, Y. Yuan, Q. Huang, Y. Cao, F. Lu, Identifying conformational changes of aptamer binding to theophylline: A combined biolayer interferometry, surface-enhanced Raman spectroscopy, and molecular dynamics study, *Talanta.* 217 (2020) 121073. <https://doi.org/10.1016/j.talanta.2020.121073>.
- [108] C. Bai, Z. Lu, H. Jiang, Z. Yang, X. Liu, H. Ding, H. Li, J. Dong, A. Huang, T. Fang, Y. Jiang, L. Zhu, X. Lou, S. Li, N. Shao, Aptamer selection and application in multivalent binding-based electrical impedance detection of inactivated H1N1 virus, *Biosens. Bioelectron.* 110 (2018) 162–167. <https://doi.org/10.1016/j.bios.2018.03.047>.
- [109] G.Q. Ying, M.J. Wang, Y. Yi, J.S. Chen, J.F. Mei, Y.L. Zhang, S.Q. Chen, Construction and application of an electrochemical biosensor based on an endotoxin aptamer, *Biotechnol. Appl. Biochem.* 65 (2018) 323–327. <https://doi.org/10.1002/bab.1610>.
- [110] H. Li, S. Song, M. Wen, T. Bao, Z. Wu, H. Xiong, X. Zhang, W. Wen, S. Wang, A novel label-free electrochemical impedance aptasensor for highly sensitive detection of human interferon-gamma based on target-induced exonuclease inhibition, *Biosens. Bioelectron.* 142 (2019) 111532. <https://doi.org/10.1016/j.bios.2019.111532>.
- [111] G. Paniagua, A. Villalonga, M. Eguílaz, B. Vegas, C. Parrado, G. Rivas, P. Díez, R. Villalonga, Amperometric aptasensor for carcinoembryonic antigen based on the use of bifunctionalized Janus nanoparticles as biorecognition-signaling element, *Anal. Chim. Acta.* 1061 (2019) 84–91. <https://doi.org/10.1016/j.aca.2019.02.015>.
- [112] J. Yu, W. Tang, F. Wang, F. Zhang, Q. Wang, P. He, Simultaneous detection of streptomycin and kanamycin based on an all-solid-state potentiometric aptasensor array with a dual-internal calibration system, *Sensors Actuators, B Chem.* 311 (2020) 127857. <https://doi.org/10.1016/j.snb.2020.127857>.
- [113] S. Eissa, A. Siddiqua, R. Chinnappan, M. Zourob, Electrochemical SELEX Technique for the Selection of DNA Aptamers against the Small Molecule 11-Deoxycortisol, *ACS Appl. Bio Mater.* 2 (2019) 2624–2632. <https://doi.org/10.1021/acsabm.9b00294>.
- [114] K.-C. Lin, B. Jagannath, S. Muthukumar, S. Prasad, Sub-picomolar label-free detection of thrombin using electrochemical impedance spectroscopy of aptamer-functionalized MoS₂, *Analyst.* 142 (2017) 2770–2780. <https://doi.org/10.1039/C7AN00548B>.
- [115] A.J. Bard, L.R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, 2nd Edition, 2nd editio, 2000. <http://elib.tu-darmstadt.de/tocs/95069577.pdf>.
- [116] Q. Hu, R. Wang, H. Wang, M.F. Slavik, Y. Li, Selection of acrylamide-specific aptamers by a quartz crystal microbalance combined SELEX method and their application in rapid and specific detection of acrylamide, *Sensors Actuators, B Chem.* 273 (2018) 220–227. <https://doi.org/10.1016/j.snb.2018.06.033>.
- [117] M.H. Ali, M.E. Elsherbiny, M. Emara, Updates on aptamer research, *Int. J. Mol. Sci.* 20 (2019) 1–23. <https://doi.org/10.3390/ijms20102511>.
- [118] T. Sakamoto, NMR study of aptamers, *Aptamers.* 1 (2017) 13–18.
- [119] H. Minagawa, K. Onodera, H. Fujita, T. Sakamoto, J. Akitomi, N. Kaneko, I. Shiratori, M. Kuwahara, K. Horii, I. Waga, Selection, Characterization and Application of Artificial DNA Aptamer Containing Appended Bases with Sub-nanomolar Affinity for a Salivary Biomarker, *Sci. Rep.* 7 (2017) 1–9. <https://doi.org/10.1038/srep42716>.
- [120] Z.R. Churcher, M.A.D. Neves, H.N. Hunter, P.E. Johnson, Comparison of the free and ligand-bound imino hydrogen exchange rates for the cocaine-binding aptamer, *J. Biomol. NMR.* 68 (2017) 33–39. <https://doi.org/10.1007/s10858-017-0112-y>.

- [121] Z.R. Churcher, D. Garaev, H.N. Hunter, P.E. Johnson, Reduction in Dynamics of Base pair Opening upon Ligand Binding by the Cocaine-Binding Aptamer, *Biophys. J.* 119 (2020) 1147–1156. <https://doi.org/10.1016/j.bpj.2020.08.012>.
- [122] L. Verdonck, D. Buyst, A.M. de Vries, V. Gheerardijn, A. Madder, J.C. Martins, Tethered imidazole mediated duplex stabilization and its potential for aptamer stabilization, *Nucleic Acids Res.* 46 (2018) 11671–11686. <https://doi.org/10.1093/nar/gky1062>.
- [123] D. Buyst, V. Gheerardijn, K. Fehér, B. Van Gasse, J. Van Den Begin, J.C. Martins, A. Madder, Identification of a pKa-regulating motif stabilizing imidazole-modified double-stranded DNA, *Nucleic Acids Res.* 43 (2015) 51–62. <https://doi.org/10.1093/nar/gku1306>.
- [124] A. Oguro, A. Yanagida, Y. Fujieda, R. Amano, M. Otsu, T. Sakamoto, G. Kawai, S. Matsufuji, Two stems with different characteristics and an internal loop in an RNA aptamer contribute to spermine-binding, *J. Biochem.* 161 (2017) 197–206. <https://doi.org/10.1093/jb/mvw062>.
- [125] E. Duchardt-Ferner, M. Juen, B. Bourgeois, T. Madl, C. Kreutz, O. Ohlenschläger, J. Wöhnert, Structure of an RNA aptamer in complex with the fluorophore tetramethylrhodamine, *Nucleic Acids Res.* 48 (2020) 949–961. <https://doi.org/10.1093/nar/gkz1113>.
- [126] S. Slavkovic, Y. Zhu, Z.R. Churcher, A.A. Shoara, A.E. Johnson, P.E. Johnson, Thermodynamic analysis of cooperative ligand binding by the ATP-binding DNA aptamer indicates a population-shift binding mechanism, *Sci. Rep.* 10 (2020) 1–10. <https://doi.org/10.1038/s41598-020-76002-8>.
- [127] A.G. Marshall, J.L. Smith, Nuclear Spin-Labeled Nucleic Acids. 1. 19F Nuclear Magnetic Resonance of Escherichia coli 5-Fluorouracil-5S-RNA, *J. Am. Chem. Soc.* 99 (1973) 635–636.
- [128] L.G. Scott, M. Hennig, 19F-Site-Specific-Labeled Nucleotides for Nucleic Acid Structural Analysis by NMR, 1st ed., Elsevier Inc., 2016. <https://doi.org/10.1016/bs.mie.2015.05.015>.
- [129] M. Chrominski, M.R. Baranowski, S. Chmielinski, J. Kowalska, J. Jemielity, Synthesis of Trifluoromethylated Purine Ribonucleotides and Their Evaluation as 19F NMR Probes, *J. Org. Chem.* 85 (2020) 3440–3453. <https://doi.org/10.1021/acs.joc.9b03198>.
- [130] M.R. Baranowski, M. Warminski, J. Jemielity, J. Kowalska, 5'-fluoro(di)phosphate-labeled oligonucleotides are versatile molecular probes for studying nucleic acid secondary structure and interactions by 19F NMR, *Nucleic Acids Res.* 48 (2020) 8209–8224. <https://doi.org/10.1093/nar/gkaa470>.
- [131] T. Ishizuka, A. Yamashita, Y. Asada, Y. Xu, Studying DNA g-Quadruplex aptamer by 19F NMR, *ACS Omega.* 2 (2017) 8843–8848. <https://doi.org/10.1021/acsomega.7b01405>.
- [132] H.L. Bao, T. Ishizuka, T. Sakamoto, K. Fujimoto, T. Uechi, N. Kenmochi, Y. Xu, Characterization of human telomere RNA G-quadruplex structures in vitro and in living cells using 19F NMR spectroscopy, *Nucleic Acids Res.* 45 (2017) 5501–5511. <https://doi.org/10.1093/nar/gkx109>.
- [133] Y. Yamaoki, A. Kiyoishi, M. Miyake, F. Kano, M. Murata, T. Nagata, M. Katahira, The first successful observation of in-cell NMR signals of DNA and RNA in living human cells, *Phys. Chem. Chem. Phys.* 20 (2018) 2982–2985. <https://doi.org/10.1039/c7cp05188c>.
- [134] P. Broft, S. Dzatko, M. Krafcikova, A. Wacker, R. Hänsel-Hertsch, V. Dötsch, L. Trantirek, H. Schwalbe, In-Cell NMR Spectroscopy of Functional Riboswitch Aptamers in Eukaryotic Cells, *Angew. Chemie - Int. Ed.* (2020). <https://doi.org/10.1002/anie.202007184>.
- [135] A.D. Gelinas, D.R. Davies, N. Janjic, Embracing proteins: Structural themes in aptamer-protein complexes, *Curr. Opin. Struct. Biol.* 36 (2016) 122–132. <https://doi.org/10.1016/j.sbi.2016.01.009>.
- [136] V.J.B. Ruigrok, M. Levisson, J. Hekelaar, H. Smidt, B.W. Dijkstra, J. van der Oost, Characterization of aptamer-protein complexes by x-ray crystallography and alternative approaches, *Int. J. Mol. Sci.* 13 (2012) 10537–10552. <https://doi.org/10.3390/ijms130810537>.
- [137] C. Schmidt, M. Perbandt, S. Klussmann, C. Betzel, Molecular characterization of a ghrelin-L-aptamer complex, *J. Mol. Struct.* 1204 (2020) 127510. <https://doi.org/10.1016/j.molstruc.2019.127510>.
- [138] J.R. Stagno, Y.R. Bhandari, C.E. Conrad, Y. Liu, Y.X. Wang, Real-time crystallographic studies of the adenine riboswitch using an X-ray free-electron laser, *FEBS J.* 284 (2017) 3374–3380. <https://doi.org/10.1111/febs.14110>.
- [139] J. Ding, M. Swain, P. Yu, J.R. Stagno, Y.X. Wang, Conformational flexibility of adenine riboswitch aptamer in apo and bound states using NMR and an X-ray free electron laser, *J. Biomol. NMR.* 73

- (2019) 509–518. <https://doi.org/10.1007/s10858-019-00278-w>.
- [140] Y.M. Chang, C.K.M. Chen, M.H. Hou, Conformational changes in DNA upon ligand binding monitored by circular dichroism, *Int. J. Mol. Sci.* 13 (2012) 3394–3413. <https://doi.org/10.3390/ijms13033394>.
 - [141] D. Zhang, J. Han, Y. Li, L. Fan, X. Li, Aptamer-based K⁺ sensor: Process of aptamer transforming into G-quadruplex, *J. Phys. Chem. B.* 120 (2016) 6606–6611. <https://doi.org/10.1021/acs.jpcc.6b05002>.
 - [142] G.R. Wiedman, Y. Zhao, A. Mustaev, J. Ping, An Aptamer-Based Biosensor for the Azole Class of Antifungal Drugs, *MSphere.* 2 (2017) 2–11.
 - [143] S. Wildner, S. Huber, C. Regl, C.G. Huber, U. Lohrig, G. Gadermaier, Aptamers as quality control tool for production, storage and biosimilarity of the anti-CD20 biopharmaceutical rituximab, *Sci. Rep.* 9 (2019) 1–14. <https://doi.org/10.1038/s41598-018-37624-1>.
 - [144] M.M. Aljohani, R. Chinnappan, O.A. Alsager, R. AlZabn, A. Alhoshani, K. Weber, D. Cialla-May, J. Popp, M. Zourob, Mapping the binding region of aptamer targeting small molecule: Dabigatran etexilate, an anti-coagulant, *Talanta.* 218 (2020) 121132. <https://doi.org/10.1016/j.talanta.2020.121132>.
 - [145] R. Das, S. Doniach, Structural Studies of Proteins and Nucleic Acids in Solution Using Small Angle X-Ray Scattering (SAXS), in: *Soft Matter Charact.*, Springer, 2008: pp. 1083–1108.
 - [146] F. Mittelberger, C. Meyer, G.H. Waetzig, M. Zacharias, E. Valentini, D.I. Svergun, K. Berg, I. Lorenzen, J. Grötzinger, S. Rose-John, U. Hahn, RAID3 - An interleukin-6 receptor-binding aptamer with post-selective modification-resistant affinity, *RNA Biol.* 12 (2015) 1043–1053. <https://doi.org/10.1080/15476286.2015.1079681>.
 - [147] K. Szameit, K. Berg, S. Kruspe, E. Valentini, E. Magbanua, M. Kwiatkowski, I. Chauvot de Beauchêne, B. Krichel, K. Schamoni, C. Uetrecht, D.I. Svergun, H. Schlüter, M. Zacharias, U. Hahn, Structure and target interaction of a G-quadruplex RNA-aptamer, *RNA Biol.* 13 (2016) 973–987. <https://doi.org/10.1080/15476286.2016.1212151>.
 - [148] F.N. Tomilin, R. Moryachkov, I. Shchugoreva, V.N. Zabluda, G. Peters, M. Platunov, V. Spiridonova, A. Melnichuk, A. Atrokhova, S.S. Zamay, S.G. Ovchinnikov, G.S. Zamay, A. Sokolov, T.N. Zamay, M. V. Berezovski, A.S. Kichkailo, Four steps for revealing and adjusting the 3D structure of aptamers in solution by small-angle X-ray scattering and computer simulation, *Anal. Bioanal. Chem.* 411 (2019) 6723–6732. <https://doi.org/10.1007/s00216-019-02045-0>.