Biophysical Characterization of Aptamer-Target Interactions

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Contents

Abstract Aptamers are single-stranded nucleic acid molecules forming well-defined 3D structures. Aptamers typically bind to their ligands with high affinity and specificity. They are capable of interacting with various kinds of ligands: ions, small molecules, peptides, proteins, viruses, bacteria, and even cells. Therefore, aptamers are in widespread use as sensor molecules or as targeting agents in diagnostics and pharmaceutics. As a prerequisite for their use in these economic high-value areas, aptamers must be studied in detail with respect to different biophysical characteristics. Of central importance are basic binding parameters of the aptamer-target interaction, such as binding affinity and kinetics. Numerous biophysical methods with different features, characteristics, and capabilities are used in the field today for this purpose.
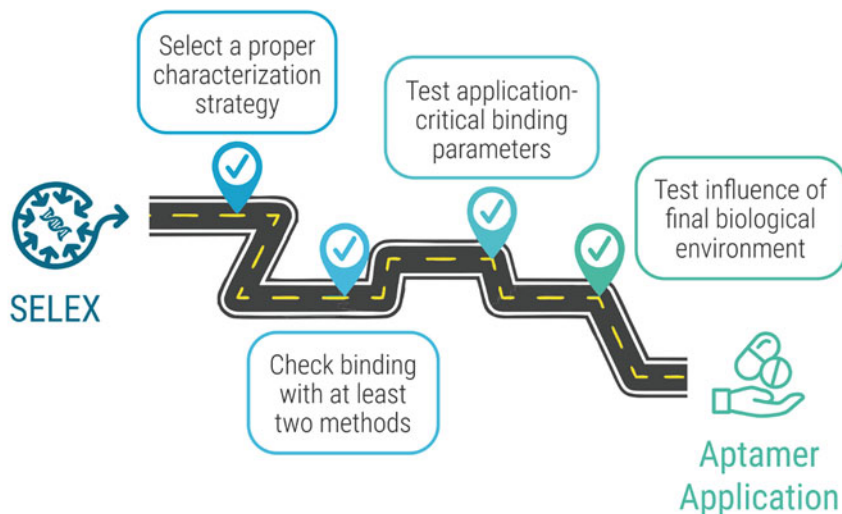
This chapter provides an overview of the current state-of-the-art technologies for studying interactions between aptamers and targets and discusses their advantages as well as drawbacks. Furthermore, essential aspects influencing any aptamer

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characterization strategy will be presented. Finally, issues of comparability of binding data between different aptamer characterization technologies will be discussed.

Graphical Abstract

The winding road to an aptamer-based application

SELEX

Select a proper characterization strategy

Test application-critical binding parameters

Test influence of final biological environment

Check binding with at least two methods

Aptamer Application

1 Basic Binding Parameters in Aptamer Development

Basic biophysical binding parameters such as affinity, kinetics, or thermodynamics are key aspects in the development of aptamers for pharmaceutical and diagnostic use. Binding affinity is a measure of binding strength between aptamer and target and is usually reported as an equilibrium dissociation constant (K_D). The lower this constant, the higher the binding strength between aptamer and target. In the course of aptamer development, affinity is often used to initially rank a pool of aptamers to select relevant candidates. In addition, affinity enables to express selectivity of

different aptamers to one target or the specificity of an aptamer to one or few of multiple targets.

Binding kinetics describes the time-dependent, dynamic component of the binding event between aptamer and target. The association rate constant k_a (k_{on}, in $M^{-1} s^{-1}$) describes the association of aptamer and target to the binary (or higher order) complex over time. The dissociation rate constant k_d (k_{off}, in s^{-1}) describes the rate of dissociation of aptamer and target and is hence a measure of temporal stability of the aptamer-target complex. In order to ensure proper functionality of an aptamer in its final application (e.g., in diagnostic use), aptamers with desired binding kinetics may be chosen during the development phase.

Binding thermodynamics describe the enthalpic (ΔH) and entropic ($-T\Delta S$) parameters of the interaction between aptamer and target, which will only occur spontaneously when the Gibbs free energy (ΔG) of the interaction is negative (either enthalpy or entropy driven). The enthalpic parameter ΔH is the energy change resulting from the formation of non-covalent interactions between aptamer and target and the changes of hydrogen bond and van der Waals interactions between aptamer, target, and the solvent. The entropic counterpart ΔS represents the global thermodynamic property of the system, hence the degree of freedom of the system. Thermodynamic parameters are thus helpful to understand the molecular principles of aptamer-target interactions and to optimize aptamers with respect to certain thermodynamic characteristics.

In the following, different biophysical methods to characterize aptamer-target interactions are described. Besides the seven well-established and broadly applied methods, Surface Plasmon Resonance (SPR) [1–5], Biolayer Interferometry (BLI) [6, 7], Isothermal Titration Calorimetry (ITC) [2, 8], Fluorescence Polarization (FP or FA) [9, 10], Flow Cytometry [11, 12], Filter-Binding Assay (FB)/Filter Retention Assay [4, 13], and Electromobility Shift Assay (EMSA) [14, 15], two emerging technologies SwitchSENSE (SwS) [16] and MicroScale Thermophoresis (MST) [3, 13, 17] are discussed.

2 Biophysical Techniques to Study Basic Binding Parameters of Aptamers and Their Target Molecules

Numerous physical and biophysical methods and technologies are available today for determining the aforementioned basic binding parameters of aptamer-target interactions. The methods differ with respect to the type of physical readout and their information content. Some determine binding parameters in an indirect manner, others by direct readout; some work in solution, whereas others require immobilization of either target or aptamer to a solid phase. Modification-free, label-free, or fluorescent technologies are available. Some methods characterize interactions in a steady-state equilibrium, whereas others analyze dynamic binding kinetics. Some techniques are capable of studying interactions between aptamers and whole cells,

and others can detect binding of the smallest ions to an aptamer. Furthermore, all available technologies have different prerequisites and requirements with respect to the sample material. Development of an aptamer for later use in diagnostics or as a therapeutic agent thus needs careful selection of the right tools and methods at the right time and for the right application.

2.1 Biophysical Principles and Readouts of Selected Techniques

Typically, a complex of two interaction partners differs from the respective individual molecules in many molecular parameters, such as size, structure, shape, energetic state, charge, or hydration shell. Biophysical technologies either directly read out these changes in order to obtain basic binding parameters or they indirectly monitor effects correlated to these changes. Table 1 summarizes the readout principles of the biophysical methods described below, as well as their key information content. Furthermore, the key advantage of each respective technique is indicated.

2.1.1 Surface Plasmon Resonance (SPR) [1–5]

SPR appears when a polarized light beam hits a metal layer (commonly a gold film) at the interface of two media with different refractive indices. Monitoring changes in refractive index upon binding of an interaction partner (analyte) to an immobilized partner (ligand) on the metal layer enables to calculate kinetic parameters (k_{on} and k_{off}) and steady-state affinity (K_D). Furthermore, thermodynamic parameters can be estimated from experimental repeats at different constant temperatures [19].

In a typical SPR experiment, one of the interaction partners is immobilized on the surface of an SPR sensor chip, whereas the other interaction partner is supplied in different concentrations via a microfluidic system. Numerous different immobilization strategies and coupling chemistries are available for both nucleic acids, proteins, peptides, and larger particles so that aptamers can be used both as the immobilized binding partner and the partner being free in solution.

2.1.2 Biolayer Interferometry (BLI) [6, 7]

BLI analyzes interference patterns of white light that is reflected from two optical layers of a sensor tip. One internal reference layer is located inside the tip and one layer at the interface between the tip and the surrounding liquid phase. Each reflection generates constructive and destructive interferences that vary with the wavelength of the incident light. Any change at the outer layer of the tip (a biocompatible surface with one interaction partner immobilized on it), for

Table 1 Readout, information content, and key advantages of selected biophysical methods

Technique	Principle	Information obtained	Key advantages
SPR	Changes in refractive index	*Affinity* (steady-state): K_D from 1 nM to 500 μM. *Kinetics*: Association rate (k_{on}) down to $1E^{-5} \, s^{-1}$ and dissociation rate (k^{off}) up to $1E^7 \, M^{-1} \, s^{-1}$. *Thermodynamics*: Binding enthalpy ΔH by Vant-Hoff (25–40°C), derived ΔG	Well-established and well-characterized technique
BLI	Changes in biolayer thickness	*Affinity* (steady-state): K_D from 1 nM to 500 μM. *Kinetics*: Association rate (k_{on}) down to $1E^{-5} \, s^{-1}$ and dissociation rate (k_{off}) up to $1E^7 \, M^{-1} \, s^{-1}$ *Thermodynamics*: Binding enthalpy ΔH by Vant-Hoff (25–40°C), derived ΔG	Offers kinetics without drawbacks of microfluidics
SwitchSENSE	Changes in movement of DNA nano-levers	*Affinity* (steady-state): K_D from 1 nM to 1 mM. *Kinetics*: Association rate (k_{on}) down to $1E^{-5} \, s^{-1}$ and dissociation rate (k_{off}) up to $1E^7 \, M^{-1} \, s^{-1}$. *Thermodynamics*: Binding enthalpy ΔH by Vant-Hoff (25–40°C), derived ΔG. Hydrodynamic radius	Fast and efficient immobilization of aptamers on the DNA nano-lever by simple sequence extension
MST	Changes in thermophoretic mobility and fluorophore microenvironment	*Affinity* (steady-state): K_D from 10 pM to 10 mM. *Thermodynamics*: Binding enthalpy ΔH by Vant-Hoff (22–45°C), derived ΔG	Ultra-low sample consumption, largest application range, and possibility to work in bioliquids
ITC	Heat changes	*Affinity* (steady-state): K_D from 1 nM to 500 μM. *Thermodynamics*: Directly binding enthalpy ΔH, derived free enthalpy of binding ΔG and binding entropy ΔS. *Stoichiometry*: Directly determinable. *Kinetics* [18]	Highest content of information. Thermodynamics directly accessible
Flow cytometry	Fluorescence changes	*Affinity* (steady-state): K_D from 10 nM to 1 mM	Only method allowing to study aptamer-cell interactions
Fluorescence polarization	Changes of polarization	*Affinity* (steady-state): K_D from 1 nM to 1 mM	Highest throughput
EMSA	Changes in molecular size and hence changes in electrophoretic mobility	*Affinity* (steady-state): K_D from 10 nM to 1 mM	Cost-efficient technique with low lab requirements
Filter-binding assay	Fluorescence changes	*Affinity* (steady-state): K_D from 1 nM to 1 mM	Rapid and cost-efficient technique

example, due to binding of a ligand, leads to different interference patterns at this reflective layer. This, in turn, causes a shift of the interference spectrum to different wavelengths. From the time-resolved monitoring of this shift, it is possible to derive real-time association (k_{on}) and dissociation rates (k_{off}) of an aptamer-target interaction. The steady-state affinity (K_D) can be extracted from equilibrium titrations. As for SPR analyses, repeat of BLI experiments at different temperatures allows for determination of thermodynamic parameters.

In a typical BLI experiment, one of the interaction partners is immobilized to the sensor tip, whereas the other partner is supplied in different concentrations in a microwell plate. As for SPR, numerous coupling methods exist that allow to analyze aptamers both as the immobilized and the in-solution interaction partner.

2.1.3   SwitchSENSE (SwS) [16]

The SwitchSENSE technology monitors voltage-driven movement of DNA nano-levers attached to a sensor surface. Usually, such a nano-lever carries one of the interaction partners by direct, covalent attachment. Binding of the other partner affects the hydrodynamic friction of the nano-lever and hence its movement on the sensor surface, which can be monitored through time-resolved single-photon counting. Kinetic parameters (k_{on} and k_{off}) and steady-state affinity (K_D) can be extracted. Furthermore, thermodynamic parameters can be estimated by analyses at different temperatures.

In a typical SwitchSENSE experiment, one interaction partner is immobilized to the nano-levers on the sensor surface, whereas the other interaction partner is titrated in different concentrations. Aptamers can often be coupled directly to the nucleic acid-based nano-levers by base-pairing.

2.1.4   MicroScale Thermophoresis (MST) [3, 13, 17]

The optical method MST is based on the combined effect of *T*emperature-*R*elated *I*ntensity *C*hange of fluorescent molecules (TRIC) and their directed movement along temperature gradients (thermophoresis). Both the TRIC effect and the thermophoretic component of the MST signal vary with three key molecular features that change upon binding between an aptamer and its target: molecular size, molecular charge, as well as the hydration shell of the molecules. Information on steady-state binding affinity (K_D) can be directly obtained from a ligand titration. By variation of assay temperatures, also thermodynamics can be determined.

In a typical MST experiment, one binding partner is held at a constant concentration and is monitored for its TRIC effect and thermophoretic movement by its intrinsic fluorescence or by a coupled fluorescent dye. The other binding partner is titrated usually in 16 dilution steps in order to sample a very large ligand concentration range. Aptamers can be used in MST very straightforward as the constant, fluorescent interaction partner, because they can be easily obtained with all kinds of

fluorescent dyes attached. Alternatively, proteins can be labeled with fluorescent dyes for MST, and an aptamer can be used as the non-fluorescent, titrated interaction partner.

2.1.5 Isothermal Titration Calorimetry (ITC) [2, 8]

The calorimetric method ITC directly measures the heat released or consumed in the course of a molecular binding event. The technology offers high information content. Besides thermodynamic parameters such as ΔH, ΔS, and ΔG, the equilibrium-binding affinity (K_D) and interaction stoichiometry can be determined from the same experiment. Recent developments even allow to study kinetics by ITC [18]. Hence ITC offers the highest information content.

In a typical ITC experiment, one interaction partner is put into a reaction cell at a constant volume and concentration, whereas the other partner is titrated into the reaction cell via a rotating syringe. ITC allows to study aptamer-target interactions without modification of the molecules.

2.1.6 Fluorescence Polarization or Fluorescence Anisotropy (FP or FA) [9, 10]

FP (often called FA) is based on the phenomenon that the polarization plane of emitted light of a small fluorescent molecule (excited with plane-polarized light) changes upon binding of an interaction partner. FP enables to calculate the affinity (K_D) of the aptamer-target interaction.

In a typical FP or FA experiment, one binding partner (the smaller one) is monitored via an attached fluorescent dye and held at constant concentration during the experiment, whereas the other binding partner is titrated across a certain concentration range.

2.1.7 Flow Cytometry [11, 12]

This optical method is commonly used to quantify the interaction strength between aptamers and whole cells, by sorting populations of cells that show interaction to fluorescently labeled aptamers, combined by quantification of the fluorescence signal coming from the aptamers. The steady-state affinity (K_D) can be derived from flow cytometry assays, in which the target cells are incubated with increasing concentrations of fluorescently labeled aptamers.

In a typical flow cytometry experiment, cells are incubated with fluorescent aptamers.

2.1.8 Filter-Binding Assay/Filter Retention Assay [4, 13]

Filter-binding assays quantify the signal of a fluorescently or radioactively labeled aptamer binding to a target that is immobilized on a filter membrane. Reading out different concentration steps of fluorescent aptamers allows for determining the equilibrium-binding affinity (K_D).

In a typical filter-binding assay, one interaction partner (usually the aptamer target) is immobilized on a membrane, whereas the second interaction partner (usually the aptamer) is titrated across a certain concentration range.

2.1.9 Electromobility Shift Assay (EMSA) [14, 15]

EMSA monitors mobility differences between complexed and unbound molecules in net-like matrices or gels, in order to determine the equilibrium-binding affinity (K_D) between the interaction partners.

In a typical EMSA experiment, a constant concentration of a labeled aptamer is incubated with increasing concentrations of its target. The mixed samples are loaded on a gel matrix in order to separate complexes from unbound aptamers by size. A fluorescent or radioactive readout allows for quantifying the interaction strength.

2.2 Sample Material Requirements of the Selected Biophysical Methods

Besides the fact that the data quality of any biophysical method is enhanced with increased purity, homogeneity, stability, solubility, and reduced aggregation tendency of the sample material, every method has its specific prerequisites and requirements to the sample material due to its specific principle and technical setup.

Surface-based methods, such as SPR, BLI, and SwitchSENSE, require immobilization of one interaction partner to the sensor surface. In SPR and BLI, various immobilization strategies are available using direct immobilization via capturing of epitopes already available on the target (e.g., via a hexahistidine tag on a protein). Alternatively, chemical processes are available for linking the target either directly (via amino acid side chains) on the sensor surface or to adaptors (such as biotin), which are then captured on a pre-coated surface. In SwitchSENSE, a target molecule needs to be modified with a DNA strand, which is then hybridized to a counterpart on the sensor surface. Aptamers may directly be hybridized via extended sequences that bind to the DNA nano-lever on the sensor surface.

In MST, flow cytometry, EMSA, FP, and filter-binding assays, one interaction partner must be fluorescent. MST can either work with intrinsic molecule fluorescence (tryptophan fluorescence in proteins or peptides, label-free MST) or relies on labeling one of the interaction partners with a fluorophore. In flow cytometry, the

Table 2 Consumption, throughput, and sample pretreatment of selected biophysical methods

Technique	Consumption	Throughput	Sample pretreatment
SPR	→ (medium)	↑ (high)	Immobilization via chemical modification or direct capturing of target epitopes
BLI	→ (medium)	→ (medium)	Immobilization via chemical modification or direct capturing of target epitopes
SwitchSENSE	→ (medium)	→ (medium)	Immobilization via DNA adaptor sequence on target
MST	↓ (low)	↑ (high)	Fluorescent labeling via chemical modification or monitoring of target-intrinsic tryptophan fluorescence
ITC	↑ (high)	↓ (low)	Extensive dialysis of target and ligand into the exact same buffer
Flow cytometry	↓ (low)	→ (medium)	Fluorescent labeling of the aptamer
Fluorescence polarization	↓ (low)	↑ (high)	Fluorescent labelling of the smaller partner
EMSA	↓ (low)	↓ (low)	Fluorescent or radioactive labeling of the aptamer
Filter binding assay	↓ (low)	→ (medium)	Fluorescent or radioactive labeling of the aptamer

Green indicates low consumption or high throughput, orange medium consumption or throughput, and red high sample consumption or low throughput

aptamer needs to be fluorescent. EMSA and filter-binding assays can be performed either with fluorescent or radioactive signal readout. Only label-free MST and ITC do not need any fluorescent modification of the interaction partners.

The following table (Table 2) summarizes sample consumption, throughput, and necessary sample pretreatment of the selected biophysical techniques.

2.3 Application Range of the Selected Biophysical Techniques

Different in vitro selection processes allow for the selection of aptamers for various target classes, starting at aptamers against the smallest molecules, such as ions. Other target classes for aptamer selection are small chemical molecules, peptides, nucleic acids, proteins, high-molecular-weight protein complexes, particles, viruses, bacteria, and even whole cells. Due to the enormous differences between target classes in size, charge, shape, and structure, there is currently no universally applicable biophysical method for studying all possible aptamer-target combinations. The limit of detection of the available biophysical methods is considerably influenced by the rather small size of aptamers (low to middle kDa range) and the size of the interaction partner (greatly varying from few Da as for ions to several MDa and more for whole cells). Consequently, different biophysical methods have to be applied for studying the different classes of aptamer interaction partners, as indicated in Fig. 1.

Given the small molecular sizes of aptamers, most biophysical technologies have their analytical optimum (dark blue areas in Fig. 1) in the size range of 1–500 kDa (of the aptamer's interaction partner). Target sizes <1 kDa are challenging, because mass changes upon complex formation are small, especially for the biophysical methods monitoring these mass changes (e.g., SPR, BLI). Methods that rely on the readout of additional parameters, such as MST and ITC, offer the possibility to analyze aptamer interactions with targets <1 kDa. The second challenge most biophysical technologies face is targets larger than 500 kDa (such as high-molecular-weight protein complexes, bacteria, or human cells). Besides others, issues arising from these targets are low mobility in solutions or matrices and high structural
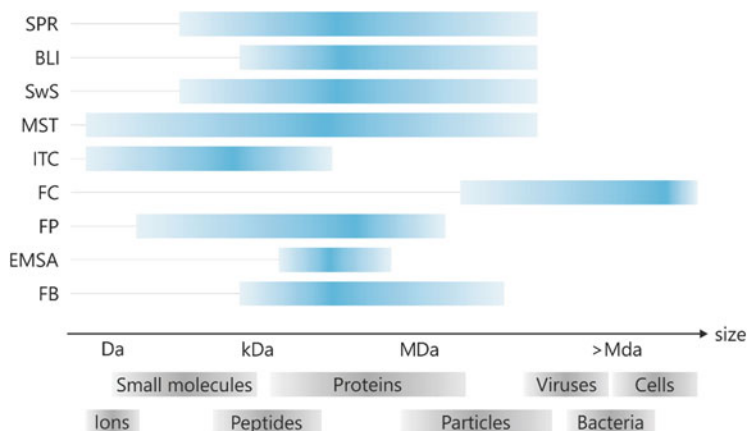

Fig. 1 Application range of selected biophysical methods for studying interactions between aptamers and different target classes. The size of the aptamer target on the x-axis increases from left to right (from ion to cells; from Da to >MDa). The application range of each biophysical method is sketched by a color gradient. Darker blue shades indicate optimal application ranges

complexity. Worth mentioning is that flow cytometry is currently the only technique enabling routing, standardized analyses of aptamer – cell interactions.

3   The Characterization Strategy: Aspects to Consider

The decision process for choosing the optimal aptamer from a pool of possible molecules requires a suitable characterization strategy. Initially, the pool of aptamers will be narrowed down to a few candidate aptamers which will be subsequently characterized in more detail. This is often done by biophysical methods with a suitable throughput, either ranking the candidates by affinity or off-rate (here MST and SPR are the most suitable techniques). Alternatively, filter-binding assays can rapidly give the required information on which aptamers bind to the target. It should be noted that in an optimal case, already at this step, the downstream application of the aptamer is considered. Choosing a therapeutic candidate aptamer, which shall later be applied systemically, can have completely different requirements at the initial selection step, compared to choosing the right candidate for a diagnostic aptamer, immobilized on a point-of-care device.

After having chosen a few initial candidates, the subsequent detailed character-ization must reflect which of the essential binding parameters – kinetics, affinity, and/or thermodynamics – are key for the later application. A fast on-rate and a slow off-rate may be most important if the aptamer needs to be tightly bound to a therapeutic target. The characterization strategy should also keep the conditions in mind at which the aptamer-target interaction will take place. Biophysical methods allowing to study aptamer-target interactions in bioliquids (e.g., human serum or plasma, bacterial cell lysate) may give important information on the behavior of a therapeutic aptamer in such complex matrices or of a diagnostic aptamer in a comparable sample. Suitable techniques for aptamer analysis in bioliquids are MST, BLI, or SwitchSENSE. In general, it is recommended to apply a minimum of two or three orthogonal methods describing the binding of an aptamer and its target, to obtain a reliable and robust view on the binding event.

Having chosen one or two suitable aptamer candidates possessing the desired binding parameters and characteristics, the next step in development is usually the elucidation of structural binding information by methods such as X-ray crystallog-raphy or NMR (not described in this chapter).

4   Affinity Constant: Estimated by Biophysical Methods

As indicated by its name, "equilibrium constant," the K_D is a natural constant describing the binding strength of two interaction partners in any possible system. Therefore, the calculated affinity values of any biophysical analytical method should theoretically be the same. However, biophysical methods can only help to obtain
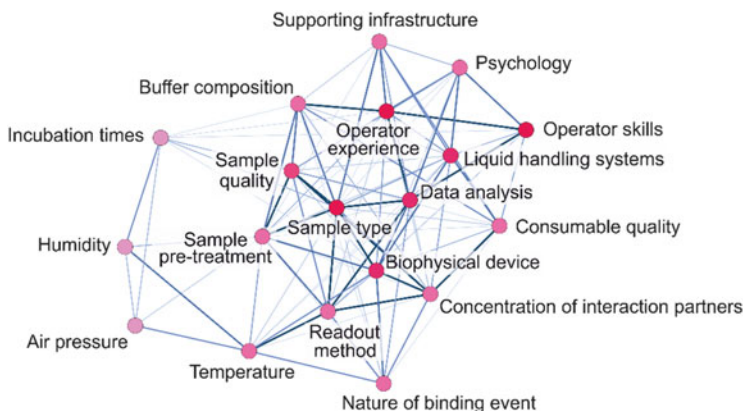
Fig. 2 Interconnectivity graph of factors influencing affinity comparability. Stronger connections and relations between factors (nodes) reflect in shorter and darker edges (connection lines) between them. Darker node colors reflect higher interconnectivity and thus factor relevance. The graph was created from a numerical representation of factor relations based on the author's opinion (Gephi, version 0.9.2 [20])

reliable estimates of this constant. It is therefore not surprising that affinities determined by different approaches may vary. In order to obtain a robust insight into the binding strength of two interaction partners and thus to approximate their true K_D value as best as possible, it is mandatory to apply different analytical approaches.

Figure 2 illustrates the interconnectivity of factors that can have considerable influence on the determination of binding affinity. Major sources of variation in affinity values are the sample type and the variability in sample quality. Origin, expression system, purification strategy, purity, homogeneity, stability, solubility, low aggregation tendency, structural integrity, biological activity, and concentration of the starting material should be as similar as possible for different biophysical approaches in order to ensure comparability.

Directly affecting sample integrity, activity, and structure are different assay-related sample pretreatments, for example, immobilization in SPR, BLI, and SwitchSENSE and fluorescent labeling in MST, FP, filter retention assay, EMSA, and flow cytometry, as well as dialysis strategy in ITC. Also, variations in the experimental setup (e.g., assay buffer composition, experiment temperature, incubation times, air pressure, or humidity) will affect sample properties. Furthermore, biological characteristics of the samples, such as oligomerization states as well as the nature of the interaction (monovalent vs multivalent interaction leading to avidity effects), will affect biophysical methods and hence the affinity determination in different manners. Please note that exact quantification of starting material (especially the titrated partner) is essential to obtain reliable affinity values.

A further considerable source of bias in affinity values originates in quantity and nature of produced data (number of data points, data point density, number of repeats, biological or technical repeats, data from kinetics or equilibrium) and how

the data are analyzed and interpreted (curve fit model, identification and treatment of outliers, statistical relevance).

Another essential factor leading to differences in K_D values are the operator's skills and experience in planning and optimizing the experimental setup, in performing the actual experiment, and in analyzing experiment data. Psychological components are often neglected: the tendency to proclaim any initially determined binding affinity as the sole benchmark for all later experiments, the ability to resist external pressure to achieve highest possible affinities, or the desire to reach benchmark affinities from literature.

Last but not least, the laboratory infrastructure and the applied scientific/industrial standards for aptamer-binding experiments (biophysical measurement device model, device maintenance, consumable quality, software version, availability of precise and reproducible liquid handling systems, and application of specific assay validation standards) can considerably influence the robustness and reliability of determined steady-state affinity values and will hence directly influence comparability of binding data.

5 Summary and Outlook

Biophysical characterization of aptamer-target interactions is an essential aspect in aptamer development. Various biophysical methods are available, each possessing specific requirements, strengths, and disadvantages. A well-planned characterization strategy is key for the success of the final application of an aptamer. Knowledge which technology to apply, how to use the technology, and how to combine and compare technologies to get the best picture of the aptamer-target interaction is the basis of a successful characterization strategy.

Current biophysical analytical methods are centered around classical experimental conditions in well-established and historically developed artificial buffer systems. Those may be beneficial for the stability of biological systems and simplify the experimental setup, as well as the interpretation of binding data. However these conditions lack biological relevance. Molecular interactions in nature do simply not occur in artificial buffer systems. The use of artificial buffer systems was historically fueled by the inability of biophysical methods to study molecular interactions in bioliquids such as serum, cell lysates, or environmental samples. Nevertheless, latest successful developments in the use of different biophysical methods such as microscale thermophoresis and biolayer interferometry indicate that a change in this paradigm is possible.

As stated in this chapter, proper characterization of aptamer-target interactions requires extensive expertise in the use of various biophysical methods, which is sometimes simply not available within the team of an aptamer development project. Lack of this expertise has already led and will result in unreliable binding data of aptamers, casting a cloud not just over a specific scientist/group, but in long term also influences the reputation of the aptamer community with all consequences.

Improving the reliability of aptamer-binding data can be achieved by integrating independent third parties (collaboration partners or other external sources) with expert knowledge in the use of biophysical methods. Scandals as in the antibody field about unreliable, unspecific, and compromised antibodies must be avoided. Consequently, the aptamer community needs to place the reliability of aptamer binding in the center of an aptamer development project, as well as in the center of any scientific review.

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