Supporting Information to Comprehensive Tutorial: Determining Small Molecule–DNA Noncovalent Binding Interactions

The methods presented in detail in the main body of the manuscript are based on the indirect observation of ligand or DNA spectral properties and thus rarely produce detailed information about structure of ligand/DNA complex or fine details about thermodynamics of complexation. For advanced studies of these types, same more elaborate experimental methods are shortly listed in Supporting Information. The molecular modelling methods, essential for understanding in detail ligand/DNA interactions, are out of scope of this experiential Tutorial.

Contents

1. Viscometry:

This method is often used as specific for classical intercalators[1] but it is characterized by low sensitivity, very limited experimental conditions and prone to false negative results due to ligand/DNA complex kinking or bending.

Principle: Classical DNA intercalators (like ethidium bromide, doxorubicin) by insertion between base pairs cause unwinding and lengthening the DNA double helix (upto 30%). This elongation increases the hydrodynamic length of the DNA molecules, which in turn increases the viscosity of the solution — something that can be detected by a very sensitive viscometer. In contrast, groove binders or electrostatic binders mostly do not cause DNA-lengthening and do not yield significant viscosity change. Therefore, a marked viscosity increase can be considered qualitative signature

of intercalation — hence the method is considered "specific" for detecting classical intercalation.[1, 2]

Experimental and results:

Measurements can be done by hand-held Ubbelohde microviscometer but are best performed with an automatic instrument with precise temperature control: (e.g. Ubbelohde micro viscometer system AVS 350 (Schott) or alternatively rolling ball system: microviscometer Lovis 2000). For the reproducible results the temperature should be ideally maintained at 25 +/- 0.05 °C. Aliquots of ligand stock solutions in aqueous buffer were added to the *c*(ds-DNA) = 5×10^{-5} M solution in the same aqueous buffer, with a ligand to DNA phosphate ratio r =0; 0.05; 0.1:0.15;0.2. Dilution should be kept as low as possible (< 5%) and should be corrected for in the calculations. The flow times were measured at least five times with a deviation of +/- 0.02 s. The viscosity index α was obtained from the flow times at varying r according to the following equation[1]:

$L/L_0 = [(t_r - t_0) / (t_{DNA} - t_0)]^{1/3} = 1 + \alpha * r$

The t_0, t_{DNA} and t_r denote the flow times of buffer, free DNA and DNA complex at ligand / DNA ratio r , respectively; L/L_0 is the relative DNA lengthening. The L/L_0 to r -plot was fitted to a straight line that gave slope α. The error in α is +/- 0.1.

Compared to more sensitive methods (e.g., fluorescence titration, isothermal titration calorimetry, circular dichroism), viscometry requires relatively large sample volumes and higher concentrations of both DNA and ligand to produce a measurable effect. Furthermore, DNA-viscometry requires high DNA purity and well-defined length, preferably >100 basepairs (polydispersity alters viscosity readings), and also strict buffer, ionic strength, and temperature conditions. Even at optimal conditions method is prone to false negatives due to the ligands that cause local distortions in DNA secondary structure — they may intercalate but introduce DNA-bends or DNA-kinks that shorten the end-to-end length of DNA instead of extending it. Also, multiple binding modes or DNA sequence dependence binding can cause false readout.

2. Electrophoretic Mobility Shift Assay (EMSA)

Principle:

EMSA detects binding between a DNA and another molecule (typically a protein,[3] but also small molecules) by observing changes in migration through a non-denaturing polyacrylamide or

agarose gel. When a small molecule binds to an oligonucleotide, it can: i) Alter the net charge of the complex; ii) Change the DNA conformation or stiffness; or iii) Increase the molecular weight. These changes slow the DNA migration during electrophoresis, resulting in a shifted band compared to unbound DNA.[4],[5] For small molecules, shifts are much less pronounced in respect to protein binding, or smears can indicate binding, stabilization, or dimerization of DNA structures such as G-quadruplexes**,** duplexes**,** or hairpins.

Experimental procedures:

- Use agarose gel (1–2%) for larger DNAs or native PAGE (typically 6–20% acrylamide) for short oligoDNA (high-percentage PAGE improves resolution)
- Run in low-salt buffer (e.g., 0.5× TBE) to reduce electrostatic screening but take care to preserve DNA folding (also ionic strength dependent). Avoid SDS or reducing agents, they could destabilize ligand/DNA complex.
- Run gels cold (4–10°C) to preserve labile structures like ligand/DNA complexes.
- Always include DNA-size ladder (see below), DNA-only, ligand-only controls (DMSO-only control if ligand is DMSO-solubilized), eventually heat-denatured DNA (to see if the shift is folded-structure-specific)
- Incubate at room temperature or 37°C for 30–60 minutes.
- DNA post-staining (SYBR Gold, SYBR Green, or for fluorophore-labeled DNA)
- Titration experiment: mix DNA (1–2 μM final) with increasing concentrations of a ligand (e.g., 0.5–10 μM) in binding buffer.
- Load equal volumes (typically 10–20 μL per lane).

DNA Size Ladder in EMSA provides a size reference for migration and helps confirm the integrity of your DNA and approximate length. For **EMSA**, the DNA size ladder is a little different from standard agarose gel electrophoresis because it needs to match the short oligonucleotide size range and native PAGE conditions (Table S1).

Table S1. Choice of DNA Size Ladders for various DNA

Oligo size range	Recommended ladder type	Example products
10–100 bp	**Short DNA ladder** (PAGE-optimized)	NEB 10 bp DNA Ladder (N3231), Thermo Fisher Low Molecular Weight DNA Ladder
50–500 bp	Low molecular weight ladder, works in agarose and PAGE	NEB 50 bp DNA Ladder (N3236)
<30 nt	Synthetic oligo ladder (custom)	Custom-made unmodified oligos mixed at equal concentration

Results:

The most common system is supercoiled plasmid DNA, which would significantly elongate only upon insertion of a ligand inside DNA, thus acting as a highly selective proof for intercalation.[5] The changes upon binding of minor groove binders are more subtle and electrostatic binding along DNApolyanionic backbone can collapse DNA structure, yielding very strong changes in EMSA. EMSA mobility of shorter oligoDNA is significantly less sensitive to ligand binding and requires careful planning od DNA length, high affinity of ligand/DNA complex ($Ks < 10^6$ M^{-1}, see 3.1 in the manuscript), and EMSA conditions.

The observed smearing above main DNA line is often not an artifact — it can indicate DNA-to-DNA stacking or dynamic dimerization, especially in G-quadruplex assays.

Under favorable conditions EMSA results can also be quantified by running simultaneously various ligand/DNA ratios (titration), which can be related to Scatchard eq from 3.1. in the manuscript.

Table S2. Pitfalls in EMSA for Small Molecule–DNA Binding

Pitfall	Possible Cause	Solution / Recommendation
No shift observed despite confirmed binding in other assays	Ligand does not alter DNA charge or size enough to affect mobility	EMSA may not detect all types of binding—use CD, UV-Vis, or melting assays as complementary methods

Pitfall	Possible Cause	Solution / Recommendation
	DNA is too short or too small to show a detectable shift	Use longer oligonucleotides (≥24 nt) or switch to PAGE for higher resolution
Smearing instead of sharp bands	Partial binding or multiple binding stoichiometries	Optimize ligand:DNA ratios; use fixed conditions for annealing; consider that smearing may reflect higher-order structures like G4 dimers
	Ligand induces aggregation or multimerization	Confirm specificity using competitor DNA; try lowering ligand concentration
Complete disappearance of DNA band	Ligand blocks DNA staining (e.g., competes with SYBR Gold or fluorescein)	Stain with a dye that binds differently (e.g., post-stain with SYBR Green); use labeled oligos for direct detection
	DNA-ligand complex is too large to enter the gel	Use lower % gels or reduce sample incubation time to avoid aggregation
Unreliable results across replicates	Inconsistent DNA folding or batch variation	Always anneal DNA under consistent conditions (95°C → slow cool); use freshly prepared DNA stocks
	Poor mixing or pipetting error in small volumes	Mix gently but thoroughly; consider scaling up sample volume slightly
Ligand-only lane shows faint bands or fluorescence	Ligand self-aggregation or autofluorescence	Run ligand-alone controls; image gels with and without DNA to subtract background
Unexpected migration patterns	Change in DNA topology (e.g., G-quadruplex folding)	Confirm folding state via CD or UV melting; differences in mobility may reflect structural change rather than mass/charge shift

Pitfall	Possible Cause	Solution / Recommendation
Gel artifacts or streaks	Salt or DMSO precipitates	Keep final DMSO below 5%; dialyze or dilute high-salt samples before loading

3. Microcalorimetry

Principle: is a powerful biophysical technique for studying the thermodynamics of small molecule interactions with DNA by direct observation of the heat released or absorbed when a ligand binds to DNA. It provides detailed information about binding affinity, stoichiometry, and the enthalpy and entropy changes associated with these interactions. This method is complemental to all aforementioned spectrophotometric methods since it does not use light and thus, avoids all related problems and pitfalls.

There are two primary types of calorimetry commonly used for such studies, nicely summarized by the one of instrument producers.[6]

3.1. Isothermal Titration Calorimetry (ITC)

Directly measures at fixed temperature the heat released or absorbed when a ligand is incrementally injected into a sample cell in which binds to DNA. It is generally assumed that ITC experiment will give a complete thermodynamic profile of interaction between the ligand and DNA in a single measurement:

Results

The binding affinity (*Ks*) and stoichiometry (analogous to Scatchard ration=[bound ligand]/[DNA]), as well as thermodynamic parameters: ΔH (enthalpy change Energy change due to bond formation or breaking); ΔS (entropy change, reflects changes in molecular disorder, such as desolvation or structural rearrangements); ΔG (Gibbs free energy, determined from ΔH and ΔS, indicating the spontaneity of binding).

Limitations: However, it is important to note that the calculation of binding parameters typically assumes that binding occurs solely between ligand and DNA. In reality, additional interactions also take place between ligand, DNA and other present ions (originating from buffer), and solvent molecules (the most prominent effect of water but DMSO, or other solvents might be present). As a result, the measured data depend not only on temperature and pressure but also on salt concentration and pH.

Thus, it is hard to predict the response: for instance, two ligands can bind to a same DNA with similar affinity and yet, due to different enthalpic and entropic contributions, the energetics driving the reaction can be quite different. Consequently, some of contributions may be of opposite sign, resulting in strong ΔH (enthalpy change) and nice set of data for one ligand, while being negligible for the other.

3.2. Differential Scanning Calorimetry (DSC):

DSC measures the heat required to increase the temperature of a DNA sample, revealing changes in thermal stability when the small molecule binds. By monitoring melting temperature (ΔT_m) shifts, this method is directly complemental to section 3.3. of the manuscript, providing more detailed insights into the thermodynamics (ΔH and ΔS) of DNA stabilization by ligand binding. Although the DSC approach is less direct than ITC, DSC can measure much higher binding constants, because DSC response depends on a DNA change (free DNA and in complex with ligand), so high-affinity ligand can be present at a very low concentration.

4. Nuclear Magnetic Resonance (NMR)

Principle: Nuclear Magnetic Resonance (NMR) spectroscopy is the most informative non-destructive technique for probing structural and dynamic aspects of ligand–DNA interactions in solution, close to biorelevant conditions. It can identify binding sites and binding modes (intercalation, groove binding, external electrostatic binding), measure structural perturbations to DNA upon ligand binding.[7] It can even be measured in living cells.[8]

The most common NMR approaches to study of ligand/DNA interactions:

a) ^{1}H NMR (Quick, allows comparatively low conc. of DNA and ligand ~ 1 - 0.01 mM): Monitors chemical shift perturbations in DNA (imino protons (10–15 ppm) and aromatic protons) upon ligand titration. Also, detects adequate changes in ligand NMR spectrum.

Results: Imino proton protection suggests ligand binding that shields DNA from exchange with water (common in intercalation and groove binding). Downfield/upfield shifts of aromatic protons help identify perturbed base pairs near the binding site.

Limitations: Overlap in spectra for large oligoDNAs and complex ligands.

b) 2D Homonuclear NMR (NOESY, TOCSY)

Measured Nuclear Overhauser Effect (NOE) cross-peaks between ligand protons and DNA protons indicate spatial proximity (<5 Å), which gives main advantage of a method: high spatial

resolution. This allows more accurate distance constraints for building 3D model of ligand/DNA binding site, enabling highly precise structure determination of a complex.

Results: Pattern of NOEs can reveal orientation of intercalator in respect to adjacent basepairs or DNAgroove-contact geometry for groove binders. Moreover, sequential NOE walks allow mapping along DNA, thus characterizing its secondary structure and eventual change caused by ligand binding.

Limitations: dedicated high magnetic field instrument recommended, spectral crowding for longer DNAsequences, essential highly experienced NMR operator for application of instrument protocols and signal assignment, needs comparatively high *c*(DNAbase)>0.1 M, which could lead to inhomogeneous sample or precipitation.

c) 2D/3D Heteronuclear NMR (HSQC, HMQC, TROSY)

By use ^{13}C - or ^{15}N -labeled DNA or ligand it is possible to assign chemical shifts for specific atoms to track subtle binding-induced perturbations, like site-specific conformational changes and hydrogen bonding.

Results: Allows work with larger DNA systems; reduces signal overlap in very complicated spectra.

Limitations: Requires expensive and time-consuming isotope labeling, dedicated high magnetic field instrument recommended, spectral crowding for longer DNAsequences, essential highly experienced NMR operator for application of instrument protocols and signal assignment, needs comparatively high *c*(DNAbase)>0.1 M, which could lead to inhomogeneous sample or precipitation.

d) STD-NMR (Saturation Transfer Difference)[9]

Instrument selectively saturates proton DNA resonances, which transfer to bound ligand, highlighting ligand protons in contact with DNA. Works with low ligand concentrations (ideally 30% ligand DNA-bound, the rest in solution); does not require DNA-isotope labeling.

Results: Allow epitope mapping by identification of bound ligand parts that are closest to DNA. Ideal for very large DNA with complex basepair composition.

Limitations: Provides qualitative, not quantitative distance data. Dedicated high magnetic field instrument recommended, essential highly experienced NMR operator for application of instrument protocols and signal assignment.

5. Raman spectroscopy

Principle: Raman spectroscopy is increasingly often used to study ligand–DNA interactions because it can directly probe vibrational modes of DNA bases, the sugar–phosphate backbone, and the ligand without labeling or extensive sample preparation. However, it's rather low sensitivity (μM–mM range) limits applicability in biorelevant conditions.

There are several techniques for increasing sensitivity, some like Tip Enhanced Raman Spectroscopy (TERS) or Stimulated Raman scattering (SRS) requiring specific, hardly available instrumentation.

Rather common, easily accessible and well-established technique is Surface-Enhanced Raman Spectroscopy (SERS),[10,11] which enhances Raman signals by several orders of magnitude (up to 10^6–10^{12}×) when analyte molecules are in close proximity to nanostructured noble metal surfaces (e.g., silver, gold). Although this is not strictly biorelevant homogeneous solution any more (sensing is happening on the metal nanoparticle surface), the obtained information is very structurally informative (reporting the changes in vibrational/rotational modes of specific structural groups of molecules) and can help in understanding of various binding modes of ligand to DNA, some of which completely remove positively charged ligand from nanoparticle surface (e.g. intercalation, dep groove binding), while other still remain visible to SERS (ligands binding to outer DNA surface, particularly to DNA backbone). Comparison of Raman and SERS in ligand/DNA sensing is summarized in Table S3.

Results

Common SERS spectral indicators in DNA–ligand binding are phosphate backbone bands (~1085–1100 cm^{-1} , shifts/intensity changes indicate electrostatic interactions), DNAbase breathing modes (~730 cm^{-1} adenine, ~1485 cm^{-1} guanine, variations may indicate intercalation or groove binding), Ligand-specific modes (e.g. groups with specific strong signals, like , polyyne bands (~2000–2300 cm^{-1})[12]; enhanced when the ligand is close to the surface, and disappearing/changing upon binding into DNA: confirming complex formation). [13]

Limitations:

i) Requires careful control of nanoparticle aggregation and surface chemistry to ensure reproducibility.

ii) Enhancement is highly dependent on the distance between the analyte and the metallic surface (~1–2 nm for strong enhancement).

iii) Interpretation can be complicated if both DNA and ligand interact with the metal surface independently.

Table S3. Comparison of conventional Raman *vs* SERS for ligand–DNA binding analysis, including pitfalls.

Feature	Conventional Raman Spectroscopy	Surface-Enhanced Raman Spectroscopy (SERS)
Detection sensitivity	μM–mM range	nM–pM range (up to single-molecule sensitivity)
Signal enhancement	Intrinsic Raman scattering only	Enhancement factors of 10^6–10^{12} via plasmonic effects
Sample requirements	Moderate concentration; larger sample volumes possible	Very small sample amounts; requires nanostructured metal substrates (e.g., Au, Ag)
Specificity for ligand–DNA complex	Both free and bound species contribute to spectra	Mostly detects molecules adsorbed near or on the metal surface, thus senses surface-bound species
Spectral resolution	High, but limited by signal-to-noise at low concentrations	Can retain high resolution while achieving high signal at very low concentrations
Structural information	Provides vibrational fingerprints for DNA, ligand, and their interactions	Same vibrational fingerprints, but stronger for modes close to surface; more sensitive to subtle binding-induced shifts
Time resolution	Suitable for slow or moderately fast binding studies	Enables rapid, even real-time monitoring of binding kinetics at low concentrations
Experimental complexity	Straightforward setup; minimal surface chemistry considerations	Requires nanoparticle synthesis or roughened substrates; control over

Feature	Conventional Raman Spectroscopy	Surface-Enhanced Raman Spectroscopy (SERS)
		aggregation and surface chemistry is critical for reproducibility
Interference risk	Minimal background if sample is pure	Possible interference from substrate signals or non-specific adsorption
Best use cases	High-concentration binding studies, bulk structural analysis, detecting major conformational changes	Ultra-sensitive detection of ligand–DNA binding, single-molecule studies, complex biological mixtures

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