How reliable is the evaluation of DNA binding constants? Insights and best practices based on an inter-laboratory fluorescence titration study

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HIGHLIGHTS

- Ethidium-DNA is chosen as a model for a study extendable to other systems/techniques.
- Fluorescence titration best practices are discussed and a protocol is proposed.
- Data fitting pros and cons using different equations and software are discussed.
- Binding constant and DNA site dimension dispersions are analysed.
- An unavoidable uncertainty of 5% (log K rsd) is evidenced.

GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT

In all experimental sciences, the precision and reliability of quantitative measurements are paramount. This is particularly true when examining the interactions between small molecules and biomolecules/polyelectrolytes, such as DNAs/RNAs, and yet it is overlooked in most publications of thermodynamic binding parameters. This paper presents findings from COST Action 18202 "Network for Equilibria and Chemical Thermodynamics

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Multi-wavelength
Scatchard plot

Advanced Research," which assessed the consistency of data derived from the interactions of calf-thymus DNA (CT-DNA) with the fluorescent intercalator ethidium bromide (EB) through spectrofluorimetric titrations. We first discuss critical experimental aspects and propose a reference experimental protocol which can be used to calibrate procedures for the determination of nucleic acid binding equilibrium constants. We then fit the experimental points according to different procedures and analyse the results focusing on the statistical dispersion of the data, aiming at enlightening the strong and weak points of different fitting procedures. The implications of this work are significant, demonstrating how the statistical dispersion of experimental data can influence the interpretation of biochemical coordination mechanisms. Our study reveals that, despite rigorous protocol standardization, the determination of binding parameters remains sensitive to the choice of data fitting method, with deviations in the logarithmic stability constant ($\log K$) values not falling below 5 % relative standard deviation (RSD), or ± 0.5 $\log K$ units for 95 % confidence. This variability evidences the critical need for standardized best practices in data treatment as well as experimental procedures. Although our study focuses on the EB/CT-DNA system through fluorescence titrations, the broader implications for other methodologies across various biochemical systems highlight the importance of this first-of-its-kind inter-laboratory comparison in advancing our understanding of biochemical coordination processes.

1. Introduction

How can one efficiently and robustly determine the value of a binding constant between a small molecule and a nucleic acid? Starting from the pioneering studies where the whole manuscript was devoted to a detailed mechanistic analysis of a single binding process, there is now a demand for faster methods to assess affinities. The associated experimental procedure and calculations appear in many papers as a simple routine. Instead, the procedure to extract numbers from experiments relies on many tricky aspects, which strongly affect the value of the K equilibrium constant calculated. Some papers discuss this topic [1–7], but more from a mathematical point of view; on the other hand, to the best of our knowledge, the literature lacks in presenting any inter-laboratory practical exercise to determine equilibrium constants. This work has four objectives: (a) to identify a system which can be used as a validation standard; (b) to perform inter-laboratory benchmarking exercises for data analysis and determination of the binding constant in the standard system; (c) to critically assess the bias sources and the data dispersion; (d) to develop a protocol, based on the standard system, to test the reliability of a procedure for small molecules-biosubstrates binding.

First step was to choose a standard system. Among the variety of possible binding modes for nucleic acids, DNA intercalation was chosen because it is both a process which attracts high biomedical interest and that, from the experimental point of view, is usually the one which produces the higher signal changes. Fluorescence was chosen because, within spectroscopic techniques, it enables high sensitivity (low concentrations to avoid aggregation processes that overlap the binding event) and it is widely used. However, our discussion may be extended to UV–visible absorption as well and the general approach even to other techniques dealing with a measured signal proportional to the concentration of the diagnostic species such as circular dichroism or calorimetric titrations.

We defined a "golden standard" fluorophore – DNA pair system based on the reactants' stability, cost, availability and ease of handling. The least expensive, most used and most widely available commercial natural double-helical DNA is calf thymus DNA (CT-DNA). CT-DNA is not adequate for site specific DNA binding studies, but it is the perfect candidate for the determination of non-specific intercalation constants. An inexpensive fluorophore was selected which may be easily purchased in high purity and is stable in solution (aggregation or aquation reactions are avoided). Toxicity should be the lowest possible (although intercalators will never be harmless). Also, a well-known system, connected to a high amount of available literature data is needed. Minor groove binders (DAPI, Hoechst dyes) were quickly excluded, since the bound dyes tend to quench the fluorescence of each other [8,9].

The initial candidates for small molecule/chromophore were cyanine dyes, porphyrins, metal-salphen complexes, ruthenium complexes and ethidium bromide (EB). Cyanine dyes show strong absorption in the visible range of the spectrum and a sharp increase in fluorescence emission when interacting with DNA. Their toxicity is not a severe issue and thiazole orange is well established as a gold standard in the indirect evaluation of the interaction of molecules to non-canonical nucleic acid structures, mainly G-quadruplexes, by displacement assays [10]. However, the literature on intercalation is not so extensive and strong auto-aggregation processes affect the data. Moreover, some cyanine dyes have poor photostability and the commercial companies do not supply them with a high degree of purity. Similarly, in the case of porphyrins, the prevalence of strong self-aggregation processes [11], the non-straightforward mode of binding to DNA [12], the scarce literature data on these systems, and the difficulty of purchasing high-purity compounds all suggest that porphyrins are not the best systems for an inter-laboratory validation. For metal-salphen (N,N'-phenylenebis(salicylideneimine)) complexes, the literature data on these systems was found too limited to ensure a strong background of experimental values to be used for comparison purposes. Moreover, another weak point is that these complexes are often studied in the form of metal complexes which can dissociate. The work of J.K. Barton [13] marks the beginning of the research on the use of chiral ruthenium complexes as DNA intercalators. However, the presence of enantiomers is a major disadvantage for these systems, as they may have different binding features. Thus, EB was chosen among all possibilities as a probe/target molecule for our comprehensive study. EB is indeed not too easy to handle and its toxicity, and 'environmentally non friendly' nature (i.e. shipping and disposal) is known. However, EB has been deeply studied and it has the advantage of being readily-available as a staining agent for many types of chemical/biological experiments in the laboratories. Many research laboratories are already organised to correctly handle and dispose of it. It can be excited and studied in the visible range, far from any DNA interference. Also, it is a very well-known dye that mainly intercalates between base pairs of DNA, it is inexpensive, provided in many forms (solid or solution in line with the different national regulations) by several different companies worldwide and its solutions are stable for long periods of time.

Numerous excellent works have been published on the nature, extent and conditions of EB – DNA binding. In this paragraph, we briefly summarize the findings most relevant to the present work. The intercalative binding mode of ethidium on double-stranded DNA was described in the early work of LePecq and Paoletti, and a secondary, presumably electrostatic, binding mode was also reported [14–17]. The two types of binding cannot be distinguished in UV–Vis measurements but in fluorometry, especially by time-resolved experiments [15,18–20]. Enhancement of fluorescence upon intercalation is attributed to a reduction in the rate of excited state proton transfer to solvent molecules [21]. The buffer composition, especially the ionic strength, strongly influences the binding strength of EB on DNA, and at high ionic strengths (e.g. 1 M NaCl) or for diluted concentrations the secondary binding becomes negligible [14,21–23]. Ethidium hardly binds to single-

stranded DNA and double stranded B-form is preferred over Z-type and G-quadruplex tertiary structures [14,24]. The dye has no explicit sequence preference and therefore the source of the DNA (e.g. Proteus vulgaris. E. coli, calf thymus with somewhat different AT/GC ratios) is not a factor [14,22]. With intercalated ethidium the so called neighbour exclusion effect applies, namely a binding site size ($\mathbf{n}$ = 1.9 – 3 bp/EB, see Table S1) must be taken into account when calculating binding constants [14,24,25]. Early publications have already drawn attention to this issue and proposed the use of neighbor exclusion model of McGhee and von Hippel where binding constant and $\mathbf{n}$ are fitted together [8,25,26]. There are other independent approaches, e.g. the method of continuous variations (Job's plot) and alternatives, to determine $\mathbf{n}$ [25,27]. According to Qu and Chaires the binding constant obtained without considering the site size can be converted into the correct constant with the help of $\mathbf{n}$ [25]. The effect of pH (between pH 3 – 11 [14], the length of DNA (1.6 – 8000 kDa [14]; 200, 500 and 4228 bp [28]) and temperature (between 23 – 37 °C [14]) are considered non-critical parameters on the binding. However, study of Chaires demonstrated that intercalation is an enthalpically driven process (ΔH in the −20 to −120 kJ/mol range) [29], therefore accurate and precise temperature control is highly recommended. Although, spectrofluorometry and UV–vis spectroscopy are the most common techniques applied on EB – DNA and drug – EB – DNA systems, DNA melting temperature analysis, viscometry, ultracentrifugation and equilibrium dialysis are also useful approaches used in this field [14,24,30].

The approach used in this work starts with a description of best practices for accurately preparing the experiment and consequently proposes an experimental protocol to be followed for DNA-EB fluorescence titrations and correct acquisition of the data. Secondly, the point of extracting binding constant from experimental data is discussed. We present a detailed analysis of possible causes for biases and dispersion of binding constant values considering experimental issues. Then, the biases and statistical dispersion of the data found with different equations and software used for data analyses are compared and discussed. Fitting methods and programmes available for free and which could be widely used are considered.

Note that the discussion done in this paper may be used to properly prepare any small molecule-nucleic acid interaction study. Also, it can be extended to absorbance titrations, and even to other techniques provided that the measured signal is proportional to the concentration of the targeted species.

Overall, we hope that this manuscript will let experienced researchers re-think and get information on the real robustness of the numbers produced, together with helping beginners with a protocol that collects best practices.

2. Experimental part and data collection

2.1. Materials

The exact materials' supplier and spectrofluorometer type may have some influence on the results and will thus be listed here for the different groups that did the experiments. The titrations were all carried out in a Hellma standard fluorescence cuvette (optical path length of 1 cm × 1 cm); all instruments were thermostated to within ± 0.1 °C.

(a) Lisbon. EB (CAS 1239-45-8) is the powder from Sigma Aldrich, BioReagent for molecular biology, code E7637. CT-DNA (CAS 73049-39-5) is the deoxyribonucleic acid sodium salt from calf thymus, type I, fibers from Sigma Aldrich, code D1501. The spectrofluorometer is a SPEX® Fluorolog (Horiba Jobin Yvon) instrument in a FL3-11 configuration. The instrumental response was corrected using a correction function provided by the manufacturer.

(b) Palermo. EB is the solution from Sigma-Aldrich, BioReagent for molecular biology, 10 mg/mL in H_2O, code E1510. CT-DNA is the

purified deoxyribonucleic acid, sodium salt, calf thymus from Merck-Millipore-Calbiochem®, code 2618-1GM. The spectrofluorometer is a JASCO FP-8300 instrument.

(c) Pisa. EB is the powder by Sigma Aldrich, code E8751. CT-DNA is the deoxyribonucleic acid sodium salt from calf thymus, type I, fibers from Sigma Aldrich, code D1501. The spectrofluorometer is an LS55 (Perkin Elmer) instrument.

(d) Szeged. EB is the powder from Sigma Aldrich, code E8751. CT-DNA is the deoxyribonucleic acid sodium salt from calf thymus, type I, fibers from Sigma Aldrich, code D1501. The spectrofluorometer is a Fluoromax Plus (Horiba Jobin Yvon) instrument.

2.2. General experimental remarks

In order to prepare the working solutions in the best way, we first of all discussed the physicochemical parameters to be carefully considered. In fact, they may affect the uncertainty of our binding constant values. Therefore, a detailed inspection of literature data on the CT-DNA system was done, which will also be useful as a subsequent comparison benchmark for our values. We have highlighted both critical parameters (salt content of the medium, temperature, quenchers) and non-critical parameters (pH, GC/AT content, length of DNA chain); for the detailed discussion see Supporting Information. We also compared the literature values for the site size ($\mathbf{n}$), first defined for intercalation by Lerman as the number of adjacent nucleic acid base pairs involved in the binding of one single intercalating molecule under saturation conditions [31]. Note that another small molecule/nucleic acid system may depend differently on the parameters cited here. However, our discussion may be useful to search for similar information in the literature and to keep in mind these aspects.

Secondly, we carefully estimated other possible weak experimental points. We collected observations and tips on the reactants involved (CT-DNA and EB), which however may be intended as a general procedure. In particular, we discussed and controlled the purity of the reagents and the way to spectroscopically evaluate their molar concentrations (the DNA content will be, all along the paper, in base pairs). The detailed discussion of these aspects is in the Supporting Information.

2.3. Check for the linearity ranges

Peculiar attention is needed for linearity ranges. Reliable quantitation of binding events requires that the optical response of the probe is linear over a range of concentrations used in the assay. Thus, the Lambert-Beer law has to be obeyed and the extinction coefficients or fluorescence optical factors must be invariant all along the titration. Non-linear concentration dependencies of absorbance and fluorescence may result from polymerisation, aggregation or simple precipitation. Also, fluorescence is intrinsically non-linear but depends on $(1–10^{-Abs})$ where Abs is the absorbance at the excitation wavelength; direct proportionality on concentration is an approximation which holds only at low concentrations/absorbances [32]. In general, quantitative analysis of the binding is not recommended when linear concentration dependence cannot be ensured. This must therefore be carefully checked, both in terms of absorbance and fluorescence response of the species analysed and in the experimental conditions chosen. The groups recorded absorbance and fluorescence data of samples containing increasing EB concentrations. All groups were in agreement that the linearity of the absorbance response for EB is obeyed at least up to 7 × 10^{-5} M. Note that, in the case of fluorescent dyes, inner filter effects may also be present, producing non-linear dependencies even in the presence of a stable monomer. This should be carefully checked as this source of error may dramatically affect the data and their analysis. Our protocol for EB/CT-DNA titrations (Supporting Information) proposes reactants' concentrations such that inner filter corrections can be neglected. In our experimental conditions (0.1 M KCl, 0.01 M HEPES, pH 7.4 buffer and excitation wavelength of 510 nm) it was found that the linearity of the

fluorescence signal response to the concentration for EB holds only up to 1×10^{-5} M (Fig. 1A). Therefore, in our experiments, we never exceeded this concentration limit. The lower range is limited by the limit of quantitation, 2×10^{-6} M, and limit of detection, 6×10^{-7} M. Obviously, measurements below the limit of quantitation are much less accurate and should not be used, whereas concentrations below the limit of detection cannot be distinguished from noise. The range depends on the excitation wavelength (may be widened by exciting at a wavelength corresponding to lower absorbance), on the light path i.e. on the exact geometry of the fluorescence cell used (here 10 mm × 10 mm), and possibly also on the instrument (detector saturation). However, note that the linearity range was found in agreement for the research groups that have contributed to this work (Fig. 1B), despite the different responses due to different instruments/instrumental setups.

2.4. Photophysical characterisation of the EB-DNA system

Fig. 2 and Table 1 collects some photophysical characteristics, focusing on the absorbance and fluorescence changes occurring as ethidium changes from the free to the DNA-bound form. In Fig. 2a, the UV–vis spectral profiles are represented in the form of molar extinction coefficients, whereas Fig. 2b shows an example of the primary fluorescence spectra collected during one of the titrations. Here, the significant fluorescence enhancement occurring to EB once DNA-bound [14] is visible. Fluorescence data may suffer some bias: (a) fluorescence intensity is in arbitrary units, and some difference in the fluorescence enhancement is foreseen for different instruments/detectors; (b) the different instruments may consider or not some auto-correction (lamp fluctuations, monochromator distortion). The latter means that, even in the presence of an attentive calibration (usually based on the Raman signals of water), a slight shift in the emission wavelengths may occur (Table 1, but see also the references in the Introduction).

2.5. The EB-DNA fluorescence titrations: An optimised protocol

In general, best practices consider performing couples of titrations where titrant and titrand are inversed. This ensures the acquisition of information on a wide range of ratios between reactants. In this case, we would like to focus our attention on an intercalation reaction which is 1:1 between small molecule and site size, being the site size composed of **n** base pairs. EB should not be a highly concentrated titrant: (i) too high excess of free EB (a moderately fluorescent species) may produce a background signal; (ii) EB excess may produce different types of binding modes due to DNA-induced aggregation on the DNA backbone as well as

external binding. For these reasons, our analysis is focused on experiments made by titrating an EB solution with increasing amounts of CT-DNA, directly in the spectrofluorometric cell. Obviously, direct titrations should also consider that time can be also a critical dimension of equilibrium measurements. In the case of the present system, the equilibrium is reached within fractions of seconds [28]; however, for not that well-characterized systems, preliminary kinetic measurements cannot be spared.

Note that some of the research groups already possessed previously collected EB/CT-DNA titration data but they were recorded either at 20 °C or 25 °C, in different aqueous buffers (TRIS, phosphate, HEPES, or cacodylate, pH range 7.0——7.4) and concentration ranges. A survey of these data immediately confirmed the information coming from the literature, i.e. strong dependence of the experimental response from the medium and temperature. Even if the experimental conditions did not seem dramatically different, they still produced a very high variability of results: on this basis, they were not considered further.

The following step was to perform inter-laboratory tests where research labs carried out new EB/DNA titrations all using 0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C. These experiments will be called **SET I**. **SET I** was shared between all research groups and analysed in search of binding parameters. However, also **SET I** suffered a somewhat low reproducibility once data fitted (see below paragraph 3.2).

On the whole, what we have observed is that a detailed protocol (same concentrations, volumes, type and number of additions, number of points) is needed to enable a real comparison of the outcomes by different laboratories. The protocol is offered in the Supporting Information. It merges the experience of the researchers, the optimal physico-chemical conditions discussed above for the here analysed system and highlights best practices. It may be useful to a beginner to experience a correct fluorescence titration, to be used as a training for other systems.

Therefore, in this work, another inter-laboratory exercise was done in which research labs carried out EB/DNA titrations using not only the same conditions (0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C) but this same operational protocol (Supporting Information). These experiments will be called **SET II**. **SET II** was also shared between all research groups and analysed to determine binding parameters. All the data collected in **SET II** are made available for others to use and analyze at https://drive.google.com/drive/folders/1E0lgG3suKpdfvu7exiRHXLvoKCs0dGhq?usp=sharing.

Fig. 3A shows examples of the experimental points collected after the normalization of the fluorescence signal and in a semilogarithmic plot [8] (Fig. 3B). The latter representation better shows the discrepancies between the collected data. The titrations are symmetrical around a
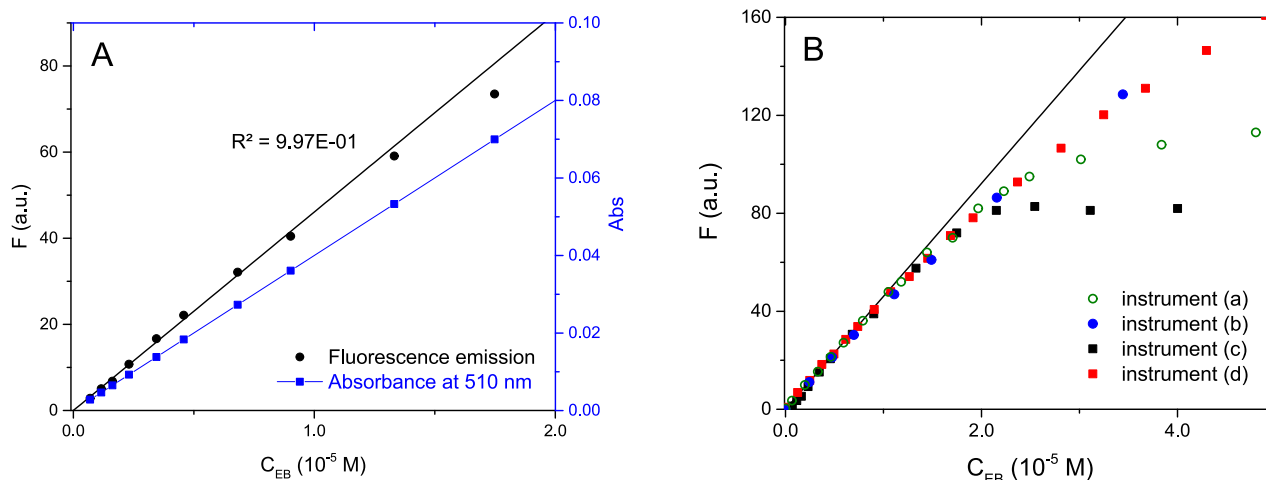

Fig. 1. Test on the linearity of fluorescence emission (F) over EB concentration – (A) range for fluorescence linear part; (B) comparison of the output of different instrument/instrumental setups (a = Lisbon, b = Palermo, c = Pisa, d = Szeged, the arbitrary scale of each instrument has been recalibrated for comparison). 0.1 M KCl, 0.01 M HEPES, pH = 7.4, 25.0 °C, λ_{ex} = 510 nm, λ_{em} = 600 nm.
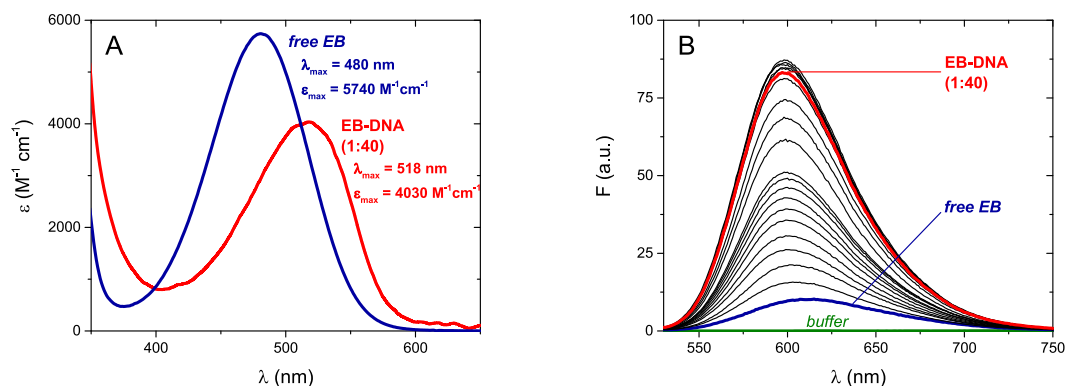
Fig. 2. (A) Molar absorption spectra of EB (black) and EB-DNA (1:40) (red) at pH = 7.4 in 10 mM HEPES, 0.1 M KCl. (B) Example of primary fluorescence emission spectra recorded for the EB – DNA titration (no correction for dilution was done here); C_{EB} = 1.84 μM; 0.1 M KCl, 0.01 M HEPES, pH = 7.4, 25.0 °C, λ_{EX} = 510 nm.

Table 1

Absorption and fluorescence spectral parameters for EB and DNA-bound E^+; 0.1 M KCl, 0.01 M HEPES, pH = 7.4, 25.0 °C.

	Abs λ(max) / ε (nm) / $(M^{-1}cm^{-1})$	Abs λ(min) / ε (nm) / $(M^{-1}cm^{-1})$	λ_{EM}(max) (nm)	Rel Int [#]
Free EB	480 / 5740	375 / 470	615 * 600 – 615	1
DNA-bound E^+ [§]	518 / 4030 [§]	405 / 800 [§]	598 * 590 – 600	14; > 19; 7; 10

[#] Fluorescence enhancement factor calculated at λ_{EX} = 510 nm and λ_{EM} = maximum emission wavelength for DNA-bound E^+ from the primary emission spectra (once dilution corrected), data are given in the order of instruments a, b, c, d; > 19 means that the very low emission for the free EB does not enable to evaluate Rel Int accurately. [§] Obtained for EB – DNA (1:40) system. * From Fig. 2B, below is the general range on the basis of this work and literature data; the binding may experimentally be seen as either no significant shift or some blue shift.

midpoint which falls around log C_{DNA}(inflection) ≈ −5. In the present case, it cannot be said that log C_{DNA}(inflection) = −log$K$ given that the relationship $C_{EB} \ll 1/K$ does not hold [8]. Also note that, for the here presented EB-DNA system, the point of the binding site **n** holds. Therefore, this matching is even less straightforward and makes the fit of the sigmoidal shape more complicated and out of the scope of the present paper. Note that, according to the conditions chosen in our work (0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C), the literature data collected in Table S1 suggest that log$K$ is close to 5.4 and bp/EB ratio is 2.5.
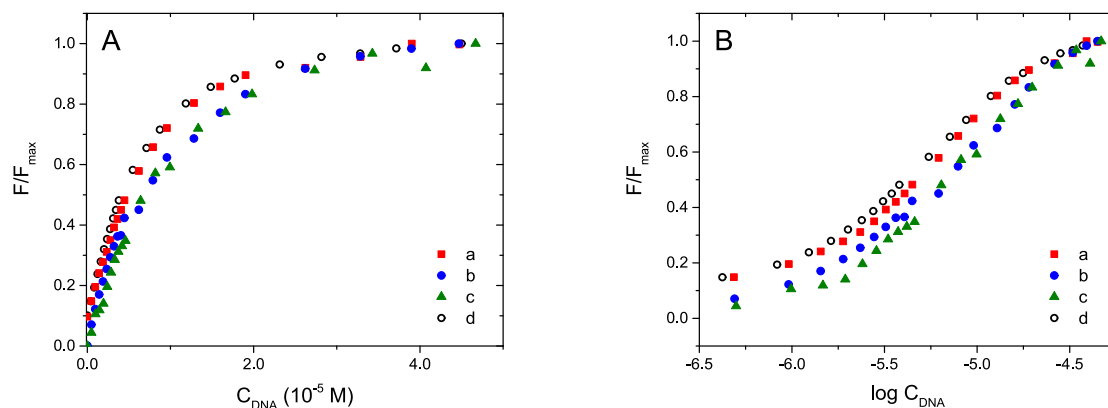
3. Data analysis

3.1. The data analysis options considered

Regarding the analysis of the data, we wanted to compare the use of "old fashioned" but still used equations (one single wavelength) to modern software which fits the data over a wide spectral range. We considered Scatchard and McGhee and von Hippel equations [1,33]: the former is outdated but was considered at least in **SET I** in the light of a possible comparison with EB-DNA pioneering studies. The weaknesses of these double reciprocal plots were highlighted in the literature [5,34,35]. However, they account for more complex features like the excluded site model and the relevant statistical dye distribution factors: these are important in systems containing long polynucleotides such as DNA. The software is certainly more powerful but fails to consider these points. In the software cases, to find the site size (**n**), the data of a whole spectrum (usually 530–750 nm) is fitted using as input the DNA concentration divided by a constant **n**. This constant is changed in successive iterations and the optimal values of **n** and K are found when the fitting error reaches a minimum. Different, freeware or not, software was considered among the more widely diffused, together with the Excel solver option.

The various options considered are (a) equations based on the excluded site model ("neighbour exclusion") developed by McGhee and von Hippel [1] and Crothers and corresponding Scatchard plot; (b) HypSpec software (http://www.hyperquad.co.uk/); (c) Bindfit free online tool (http://app.supramolecular.org/bindfit/); (d) KEV free online tool (https://k-ev.org/); (e) SPECFIT software; (f) Microsoft Excel Solver.

In order to estimate the binding constant, the point is always to

Fig. 3. Binding isotherms at maximum emission wavelength for EB/DNA titrations from different research labs (**SET II**). (A) Signal normalized to the maximum F; (B) same as A in a semi-logarithmic plot; C_{EB} initial value is ca. 2 μM, C_{DNA} is in base pairs; 0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C, λ_{EX} = 510 nm, λ_{EM} = 600 nm.

extract from the experimental data an evaluation of the fraction of free and bound EB. Modern software like HypSpec use non-linear least square fitting of the spectral data. The preliminary knowledge/estimation of the equilibrium concentrations is not necessary, the software calculates them together with the individual (fluorescence or absorbance) spectra and binding constant. The background and procedure of calculation with HypSpec is shown in the Supplementary Material. In the case of single-wavelength equations the amount of bound fluorophore is obtained as $[DNA\text{-}EB] = \Delta F / \Delta \varphi$ where $\Delta F = F - \varphi_{EB} C_{EB}$ is the signal change, $\varphi_{EB} = F°/C°_{EB}$ and $\Delta \varphi = \varphi_{DNA\text{-}EB} - \varphi_{EB}$ is the amplitude of the binding isotherm $\Delta F / C_{EB}$ vs. C_{DNA}. The evaluation of $\Delta \varphi$ is, together with the choice of the points to use, the weak aspect of the process, in particular, if the final plateau is not perfectly reached.

HypSpec (b), KEV (d), and SPECFIT (e) are based on a systematic approach to any set of equilibria. This is the application of the fundamental principles of chemical equilibrium and mass conservation to build a set of equations which can be solved numerically to determine either the concentrations of species or the equilibrium constants in systems containing any number of simultaneous equilibria. SPECFIT/32™ (Windows XP) was developed for commercial distribution by Dr Robert A. Binstead, Spectrum Software Associates, under license from Prof. Zuberbühler; it provides a global analysis of equilibria with Expanded SVD and nonlinear regression modeling by the Levenberg-Marquardt method and its calculations have been described in detail and adapted to Matlab [36,37]. HypSpec was developed by Gans et al. and explored commercially with working versions up to Windows Vista. KEV is a free software built in the R language, available for download from GitHub. It can be used online from any operating system and without installing any software. All three programs provide numerical non-linear least square minimization solutions to chemical equilibria systems, with small differences. All are adequate for absorption spectroscopy data and can be used for fluorescence data. All provide the possibility of weighted least squares. The main difficulty associated with the numerical approaches is that convergence is not always possible and depends on the system, the quality of the data and the initial approaches. It may be necessary to repeat the calculations several times starting with different initial estimates before an acceptable convergence to an acceptable result can be reached. Bindfit (c) is an online solution developed by Thordarson [35] that simplifies the set of equations for the case of one type of equilibrium only, the binding of a guest to a host molecule. This simplified set of equations can be solved in Microsoft Excel (f) by making use of the Solver Add-in. The Solver tool enables the optimization of the adjustable parameters (K, $\mathbf{n}$ and limiting signal intensities, i.e., R_f and R_b) by minimizing the sum of squared residuals $\chi^2 = \sum (R_{obs} - R_{cal})^2$ where R_{obs} correspond to the experimental signal titration data and R_{cal} correspond to the calculated data using the selected mathematical model. When the concentration of DNA is expressed in base pairs units, R_{cal} can be calculated from

$$R_{cal} = R_f + \frac{(R_b - R_f)}{2[EB]_0}\left[\left(\frac{[DNA]_0}{n} + [EB]_0 \right.\right.$$
$$\left.\left. + \frac{1}{K}\right) - \sqrt{\left(\frac{[DNA]_0}{n} + [EB]_0 + \frac{1}{K}\right)^2 - 4\frac{[DNA]_0}{n}[EB]_0}\right]$$

where R_f and R_b correspond to the spectroscopic signals of the free and bound dye, respectively [38]. This approach will be henceforward mentioned as the "solver approach".

Table S4 of the Supporting Information concisely collects information, fitting function and advantages/disadvantages of each of the fitting options. Figs. 4 and 5 show examples of the fittings performed in this work.
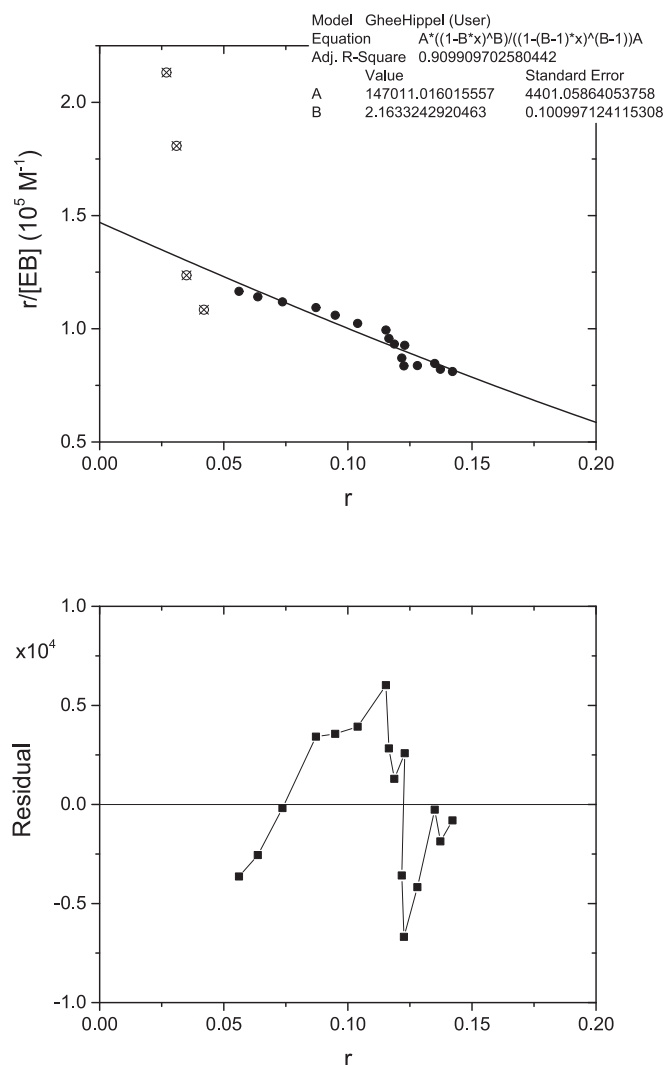

Fig. 4. Example of McGhee and von Hippel plot (upper panel) and relevant residual plot (lower panel); here, r = [EB-DNA]/C_{DNA} is the fraction of bound nucleic acid, [EB] = CEB – [EB-DNA] is the free EB content, [EB-DNA] for each of the points of the titration can be calculated as $[EB\text{-}DNA]_i = (F_i - F_0)/\Delta \varphi$ where F is the i-th fluorescence read (corrected for dilution), F_0 is the read at zero addition (free EB only) and $\Delta \varphi = \varphi_{EB\text{-}DNA} a \varphi_{EB}$ −is the change in the optical factors that can be evaluated from the plateau of the binding isotherm $\Delta F/C_{EB}$ vs. C_{DNA} [39]. SET II, C_{EB} = 2.03 μM, 0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C, λ_{EX} = 510 nm, λ_{EM} = 610 nm, the open crossed points were disregarded;. Data correspond to those in Table 3: GH-(a;C).

3.2. Data fitting results for **SET I** − same buffer

SET I is composed by titrations performed by the different groups using the same buffer (0.1 M KCl, 0.01 M HEPES, pH 7.4) and at the same temperature (25.0 °C). They produced the results shown in Table 2. They are more homogeneous than the ones produced by the collection of titrations performed in different buffers but were still considered to be not sufficiently consistent. Note that we are now referring to K values and not to $\mathbf{n}$ as this additional parameter increases the possible bias. Parameter $\mathbf{n}$ will be considered at the level of **SET II** only.

3.3. Data fitting results for **SET II** − same protocol

In **SET II** the research groups did the titrations under the same conditions as before (0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C) and
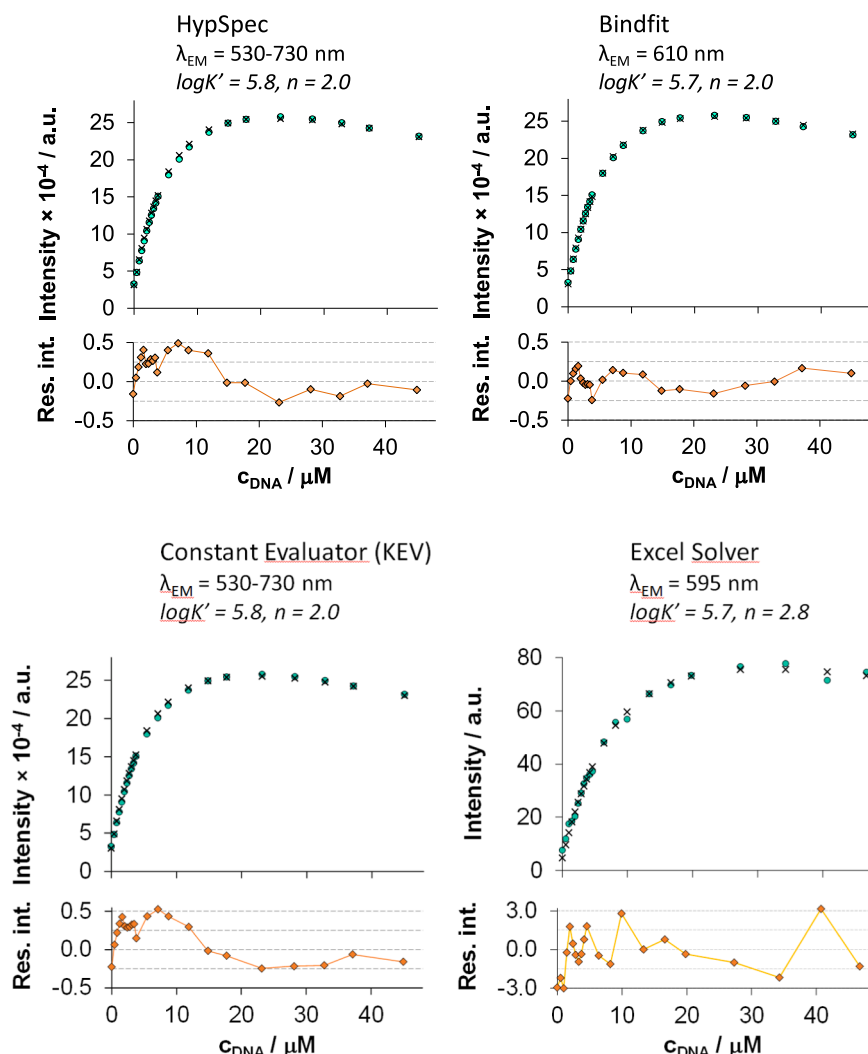
Fig. 5. Examples of measured (●) and fitted (×) fluorescence intensities shown at λ_{EM} = 610 nm together with the residual plots computed with the indicated software and parameters (log$K$ and **n**) providing the best fit, λ_{EM} indicates the data range used for calculations. **SET II**, C_{EB} = 2.03 μM, 0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C, λ_{EX} = 510 nm. Data correspond to those in Table 3: Hyp-(d;D), BF-(d;D), KEV-(d;D), EX-(c;H).

Table 2
SET I − Results for log$K$ evaluation of data fits for the titrations on the EB/CT-DNA system performed by different research groups (a, b, c or d – see 2.1) and analysed by different research groups (A, B, C, D, E) and fitting procedures (Sc = Scatchard plot, GH = McGhee and von Hippel plot, Hyp = HypSpec software); 0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C. A = Lisbon, B = Palermo, C = Pisa, D = Szeged, E = Algarve, F = Burgos.

	A Sc	C Sc	D Sc	E Sc	F Sc	B GH	C GH	F GH	C Hyp	D Hyp
a	6.78	6.09	6.82	6.31	6.57	6.89	6.68	4.77	6.23	6.50
b	6.48	6.02	5.99	6.60	5.61	6.98	6.06	5.12	6.11	6.30
c	5.54	5.43	5.40	5.67	5.33	6.28	5.12	4.84	5.30	5.32
d	6.00	5.78	6.12	5.76	5.33	6.34	5.66	4.54	5.82	5.92

following the same protocol provided in the Supporting Information. Table 3 collects the numbers obtained by analyzing the data by the different approaches listed above. As specified before, for **SET II** we also considered the **n** values. It is worth pointing out, that the **n** value is directly obtained in the case of McGhee and von Hippel and Solver − Microsoft Excel analyses. In the case of the software and online tools, the **n** value is extracted considering the C_{DNA} expressed in reaction sites (C_{DNA}(sites) = C_{DNA}(base pairs)/**n**) which gives the best fit of the fluorescence data.

4. Discussion

4.1. Statistical analysis

The dispersion or variability of results obtained in an inter-laboratory comparison study can be assigned to the combination of random effects inside each laboratory with random and possibly systematic effects between laboratories. Dispersion is commonly measured as a standard deviation (sd) or a relative standard deviation (rsd), but these parameters are only useful for large numbers of points. For relatively small groups a more intuitive parameter is the range, which is the distance between the lowest and the highest results.

Table 3

SET II – Results for logK and **n** evaluation (**n** in brackets) of data fits for the titrations on the EB/CT-DNA system performed by different research groups (a, b, c or d) and analysed by different research groups (A, B, C, D, E) and fitting procedures (GH = McGhee and von Hippel plot, Hyp = HypSpec; BF = bindfit, KEV = KEV, SF = SPECFIT, EX = excel solver); 0.1 M KCl, 0.01 M HEPES, pH 7.4, 25.0 °C and using the same protocol (Supporting Information). B = Palermo, C = Pisa, D = Szeged, G = Orléans, H = Lisbon-NOVA.

	B GH	C GH	C Hyp	D Hyp	B BF	C BF	D BF	C KEV	D KEV	G SF	C EX*	H EX
a	5.8 (1.8)	5.2 (2.2)	6.0 (3.4)	5.8 (2.7)	5.2 (1.9)	5.4 (1.0)	6.0 (3.3)	5.9 (3.2)	5.8 (2.6)	6.0 (1.0)	5.7 (1.4)	5.6 (2.9)
b	5.4 § (1.9)	5.4 (1.6)	5.8 (3.2)	5.7 (2.5)	5.1 @ (2.2)	5.3 (1.0)	5.8 (2.8)	5.9 (3.0)	5.6 (2.0)	5.8 (3.0)	5.2 (1.0)	5.4 (2.1)
c	4.9 (1.7)	5.0 § (1.3)	5.8 # (3.8)	6.0 (4.0)	5.4 (2.7)	5.2 @ (1.0)	5.4 (2.0)	5.9 & (4.0)	5.8 (3.0)	5.1 (2.7)	5.7 (1.6)	5.7 (2.8)
d	5.3 (2.5)	5.0 (1.9)	5.7 (2.0)	5.8 # (2.0)	5.8 (2.2)	5.5 (1.0)	5.7 @ (2.0)	5.8 (2.3)	5.8 & (2.0)	5.8 (4.0)	5.5 (1.1)	5.9 (2.5)

* here n is obtained by correcting C_{DNA} by the f(r) function according to McGhee and von Hippel. The symbols (§, #, @, &) enlighten the values obtained when teams fitted their own data with a common fitting function/algorithm.

The results of the statistical analysis are collected in Figs. 6 and 7. We did a first inter-comparison study (**SET I**) where all laboratories used the same experimental conditions. In **SET I** we observed an overall rsd of 10 %, which appears to be within the limits of criteria commonly used when comparing results obtained by different laboratories. But when we look at the range in logK units (see Fig. 6A − I), this is 2.4 units (from 4.5 to 6.9), too large to be acceptable. The most reasonable justification was that the different experimental procedures followed by each laboratory, including the number of points collected, exact range of concentrations, dilutions, etc, accounted for most of this variability. Therefore, we developed a detailed written experimental protocol aiming to minimize any experimental variability. In the second inter-comparison round (**SET II**) all labs followed the same protocol. This resulted in a greatly improved variability, a 5.5 % rsd and a range reduced to 1 (Fig. 6A − II). Nevertheless, it was still higher than expected for a standard procedure to be adopted as a reference standard in the determination of binding constants. Furthermore, large differences were clear between groups applying different calculation methods to the same datasets (Fig. 7B), whereas no systematic dependencies are evident in the case of the laboratory doing the experiment (Fig. 7A) or the research group doing the calculation (Fig. 7C). Analysis of variance can be used to separate the effects of different factors causing data variability. If the variability caused by one factor is significantly higher than the residual random error, then this factor is probably adding a systematic bias to the overall dispersion. Using a two-factor analysis of variance where one factor is the laboratory doing the experiment and another is the calculation method, it was possible to infer that in **SET II** no biases are coming from laboratories, but there is a bias from the calculation method. These contributions can be quantified as rsd of 6 % from laboratories and 9 % from calculation methods (see SI Table S5). The residual error, not assigned to any factor, was 4 %. Going back to data from **SET I** and doing the same analysis (Table S6), this revealed that both sources of variability were higher than in **SET II** and higher than the residual error. The residual error, 4 %, was equivalent to the one from **SET II** but this time the laboratory factor was the largest and there were significant differences in results from different laboratories. This supports the conclusion that the adoption of the same protocol was successful towards more consistent results. Indeed, the graphical comparison of the two data sets
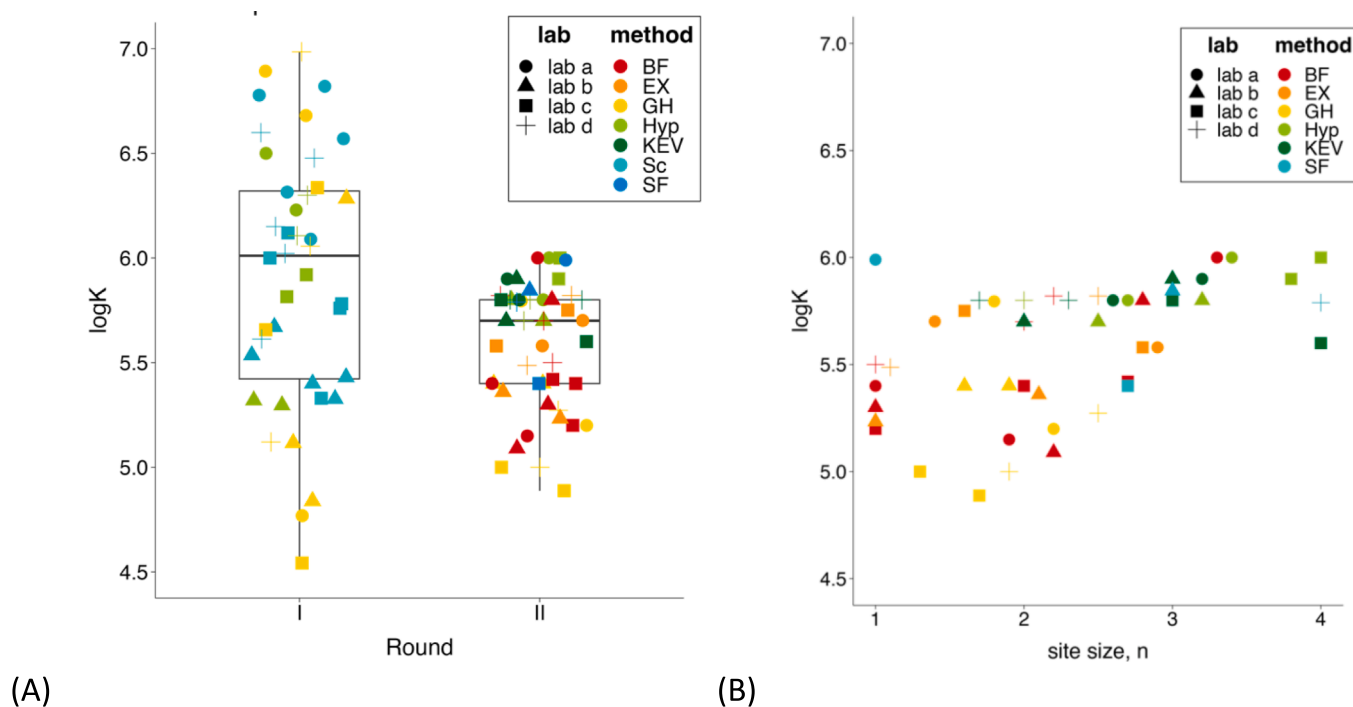

(A)   (B)

Fig. 6. (A) Dispersion of logK values obtained in the first (**SET I**) and second (**SET II**) rounds of inter-comparison. (B) Dispersion of site size values (**n**) obtained from SET II.
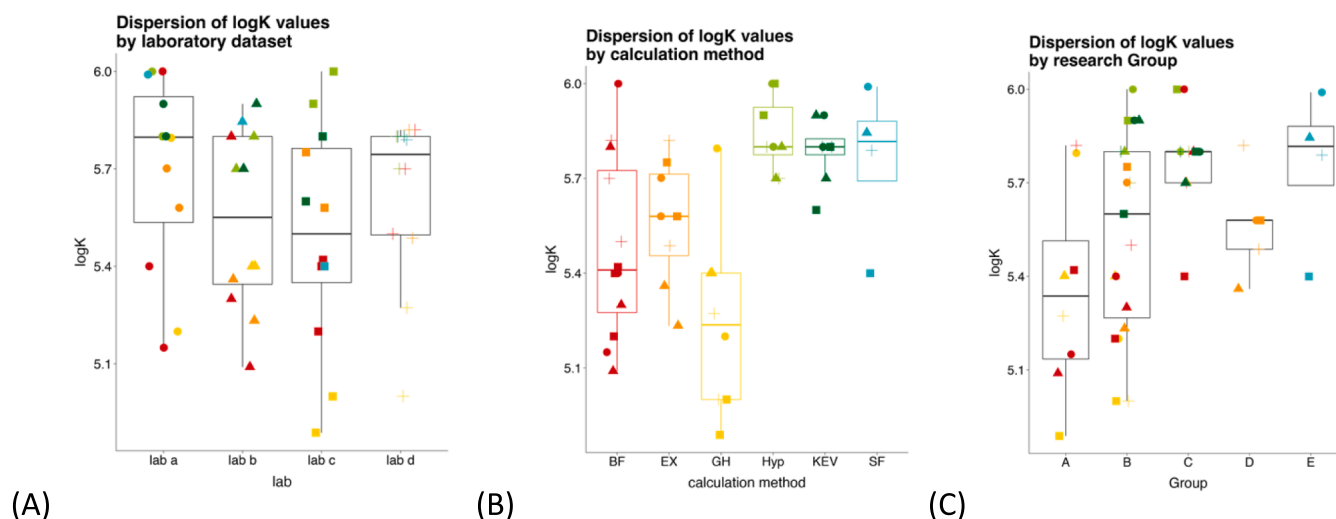
Fig. 7. Dispersion of log*K* values organized by lab dataset (A), calculation method (B) and research group doing the calculations (C). Only data obtained in **SET II** is represented. BF: Bindfit online calculator; EX: MS Excel solver; GH: MacGee-von Hippel + Scatchard plot; Hyp: HypSpec software; KEV: KEV online calculator, SF: SPECFIT software.

(Fig. 6A) clearly shows the improvement achieved in consistency of results when a detailed common protocol was adopted by the participating laboratories (**SET II**) instead of each applying its internal procedure, even under the same experimental conditions (**SET I**).

Fig. 6B and 7B show that values derived from systematic methodologies such as KEV, Hyp, and SPECFIT consistently yield higher log*K* values and higher **n** compared to those obtained using the GH approach. The values of log*K* obtained by these methods are also less dispersed and have the same median, 5.8, albeit significantly higher than the value assumed from the literature, 5.4. The other methods give results closer to the literature reference, but more dispersed.

4.2. Discussion on the strong and weak aspects of each data analysis approach

Scatchard and McGhee & vonHippel models **PROS** The model, that takes into account occupancy of multiple binding sites, is called excluded site model ("neighbor exclusion") and an extended form of this model introduces a cooperativity factor (ω) to account for the interaction between bound drug molecules, with ω > 1 corresponding to cooperative and ω < 1 to anti-cooperative binding. The neighbor exclusion model contains the correction factor f(r) which accounts for statistical effects related to possible bound molecule rearrangements, whose extent is a function of DNA saturation degree (r). Overall, this model fits the complexity of the EB/DNA system. **CONS** In Scatchard plots two problems arise: (i) the points linearization (evaluation of the final plateau for the calculation of free and bound fractions), and (ii) the choice of the points to be used. This approach uses single wavelength data (even if in principle it is possible to use more than one wavelength with Excel solver). These equations, based on DNA saturation degree, can only be applied to titrations where DNA is added to EB and calculations need the plateau to be perfectly defined for an evaluation of the optical parameters related to the bound EB species only. The choice of points to include is crucial and not easily reproduced.

HypSpec Software and KEV **PROS** HypSpec is a widely applied software in our community. HypSpec and KEV use the Newton-Raphson iterative procedure where many wavelengths and different titrations can be fitted at the same time. The titrations can be carried out in both ways (EB titrated by DNA or *vice versa*) affording a more extended range of ratios to be considered, with possible higher accuracy of **n** determination. Both software apply a systematic approach to solve equilibria which, in theory, can be applied to any system [37,40]. Another

software applying the same procedure as HypSpec to find a binding constant is PSEQUAD. It provides the same result within 0.02 log*K* units as the former one, but it is cumbersome to use due to the lack of a graphical data entry interface [41]. **CONS** HypSpec is not freeware. Also, there is uncertainty about the future of this software and its upgrades to meet new hardware standards. The software does not enable the correction for the f(r) function (which contains **n**). The procedure involves the division of the DNA concentration by **n** and the search for the minimum of the experimental-calculated fit parameters, which is sometimes difficult due to the absence of sharp minima. Different weighting algorithms are present which may produce different outcomes.

Bindfit **PROS** It is freeware, and available online. It uses an iterative procedure where several wavelengths can be fitted at the same time. The titrations can be done in both EB/DNA or DNA/EB ways, affording a more extended range of reactant ratios in the calculations. **CONS** It has no correction for site rearrangement statistical factors. The determination of **n** takes place the same way as for HypSpec and KEV. The user needs to tick or untick the "dilution correction" and "initial value subtraction" options and these may mislead inexperienced users. Table S7 of the Supporting Information emphasizes the effects of the different entries on the log*K* numerical evaluation. It is found that the initial value subtraction option does not yield significantly different results, whereas the wrong selection of dilution correction changes log*K* by ca. 0.2 units. The online tool may no longer receive support in the future. To address this concern, one potential solution is to implement the Thordarson equation in alternative software [3]. BindFit operates only on simple systems (1:1 or 1:2 stoichiometry, single wavelength, single titrations).

SPECFIT **PROS** It was widely used in the past. It uses singular value decomposition and nonlinear regression modeling by the Levenberg-Marquardt method. It has a graphical interface showing concentrations and molar absorption coefficients of species. It involves a global fitting approach applicable to equilibrium and kinetic systems involving one or more absorbing species. It allows to simultaneously analyse multi-wavelength datasets **CONS** It is not available for present operating systems. It does not specifically calculate **n** (in DNA/EB analysis).

Microsoft Excel Solver **PROS** It is widely available. It is easily handled, customized and improved. The titrations can be done in both EB/DNA and/or DNA/EB ways, affording a more extended range of reactants ratios to be considered, and analysed simultaneously using global fitting approaches. Corrections for f(r) may be added, more easily than in other software. **CONS** This approach in principle uses the data at

a single wavelength (even if the creation of an Excel solver file that uses more than one wavelength would be possible). Some tests to add f(r) correction changed the log$K$ values in a non-unique way (decreasing or increasing it) and yielding lower **n** values so that they become farther from the 2.5 reference (Table S1): doubts arise on the efficacy of this correction. When the adjustable parameters are correlated, it may be difficult to find the global minimum. In these cases, different (non-unique) sets of parameters can be obtained depending on the initial approximations. To overcome this problem, more advanced approaches using algorithms such as simulated annealing or Monte Carlo should be envisaged to find the global minimum by efficiently exploring the multidimensional space generated by the fitting parameters.

The superiority of global systematic numerical approaches, such as Bindfit and also HypSpec and KEV, over linearization approaches, such as the McGhee and von Hippel method, has been thoroughly argued in the literature [5,34,35]. Because they make use of all spectral data and do not require simplifications or any subjective choice of linear points we expect these models to provide more accurate results. This appears to be the case in Fig. 7B, where HypSpec, KEV and SPECFIT show more consistent values of log$K$ and the same median, 5.8, while the values of **n**, estimated by optimizing the fit as a function of **n**, are less consistent. Nevertheless, these approaches cannot easily take into account the size exclusion effect introduced by McGhee and von Hippel. The solution of dividing C$_{DNA}$ by **n** to estimate the number of available binding sites in the DNA chain is unrealistic because this number, according to the exclusion model, depends on the level of saturation and thus is not constant along the isotherm. A global systematic numerical calculation is needed where **n** can be an additional fitted parameter and the equations are adapted to the neighbor exclusion model. Such an approach raises the complexity of the system and makes convergence into a single solution harder.

5. Conclusions

In this work, different groups, participants of the European COST Action 18202 – NECTAR, often with long-lasting expertise in the evaluation of DNA binding constants, have collaborated to produce an inter-laboratory exercise. Many researchers all over the world are involved in the determination of DNA binding constants and various papers have evidenced the weaknesses of some data analysis procedures. However, to the best of our knowledge, this is the first time that such a practical test has been carried out, allowing us to achieve at least three main results.

First, this paper joins together the experiences of different colleagues, so to provide tips on what to check to obtain a reliable experiment. In fact, in the first part of the work, a discussion on experimental sources of error and linearity ranges is provided.

Second, it offers less experienced colleagues a protocol (see Supporting Information) to perform a correct spectrofluorimetric titration by using the EB/CT-DNA system as a "golden standard". Our protocol proposes reactant concentrations for which linearity is ensured and inner filter corrections can be neglected. Interested researchers may practice the protocol and, then, use this experience for other systems. Also, they may test their ability with the different data analysis procedures and compare the numbers obtained with those presented here. Furthermore, the protocol can be used as a reference to reduce experimental variability and improve the comparability of results between different laboratories.

Third, this work clearly shows, also to the more experienced scientists, different interesting aspects of data analysis and precision of the numerical values extracted. The purity of the reactants is not a significant bias source. On the other hand, the use of different procedures (reactants preparation, concentrations used, number of points collected) can yield significantly different log$K$ values, even if the salt medium/buffer and the temperature are identical (**SET I** – Table 2). The more substantial variations in outcomes arise when employing diverse data

analysis methods (**SET II** – Table 3). Therefore, it is demonstrated with a practical test that (and to what extent) data analysis by different operators is dramatically important. This is especially true in the case of DNA, which is a complex system (polyelectrolyte) and for which two parameters (binding constant K and site size **n**) need to be simultaneously considered. As dispersion sources, we recall that the software may also include some correction/weighting functions that are differently managed by the user. Also, the evaluation of the final plateau for the calculation of free and bound fractions and choice of points are critical aspects in the case of single-wavelength equations. The site size **n** suffers a very high error, possibly because the different software does not consider it directly and that it was therefore evaluated indirectly (from the minimum error for the fitting parameters, which is arbitrarily decided by each researcher). The log$K$ value is more precise, with ± 5 % (rsd). Overall, the variability is much higher between calculation methods than between laboratories. By inspecting Fig. 6B and 7B it could be speculated that HypSpec, KEV and SPECFIT return slightly higher log$K$ and **n** values with respect to other procedures. However, our data do not enable us to assess the better or worse approach. Additional experiments would be needed to better investigate and prove these details. However, we showed that the values fitted with an old program (for instance SPECFIT) fall into the range obtained with other programs. Also, there is no striking difference between the performances of software or an "old-fashioned" equation based on a single wavelength.

Overall, there is a non-negligible and unavoidable uncertainty in the values that cannot be lower than 5 % (rsd), which translates to an uncertainty of ± 0.5 log units for 95 % confidence. A 5 % error between laboratories for a complex procedure such as DNA binding is not so bad, considering all factors involved. On the other hand, the high possible variability shows how urgent it is to agree on standard methods for the determination of K values which carefully consider the fitting model and do inter-comparison studies such as the one presented here. On this basis, another final take-home message is that, whilst it can be safe to produce a reactivity scale for different systems studied in the same laboratory and analysed through the same procedure, the greatest care needs to be applied when comparing binding parameters by using literature data.

Declaration of competing interest

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Author Contributions

All authors contributed to conceptualization, formal analysis, methodology, investigation, writing – review & editing. O.D. was particularly involved in investigation and methodology, connected to optimising the protocol for performing the experiments. T.B. was particularly involved in supervision and writing – original draft.

Appendix A. Supplementary data

Discussion on the physicochemical parameters affecting the EB/DNA interaction strength including collection of literature data on EB/CT-DNA binding; experimental aspects affecting the correct evaluation of the EB/DNA binding including tables with collection of molar extinction coefficients; background and procedure of calculation with HypSpec and other software; comparison of the respective methods used frequently for calculation of binding constants in macromolecule-small ligand systems; statistical details and ANOVA tests for SET I and II; effect of dilution correction and initial value subtraction options on the evaluation of log*K* according to Supramol/Bindfit website software facility; practical protocol for fluorescence DNA titrations according to best practices. Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2024.125354.

Data availability

A link to a drive with raw data is provided in the text

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