The Evidence of Cooperative Binding of a Ligand to G4 DNA

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1. Introduction

In the past decade, a great research effort around four-stranded guanine-rich DNA sequences known as G4-quadruplexes (G4) [1] and small molecule ligand interactions was motivated as both innovative basis for the design of novel inhibitors of telomerase activity [2] and selective fluorescent probes [3]. Recent bioinformatics studies have shown that in the human genome there are approximately 350,000 sequences that can potentially form G4 structures [4]. The current interest in G4 interactions is based on the concept that small molecules binding would stabilize these structures’ formation, which causes an inhibition of telomerase activity, which plays a role in the immortal growth of cancer cells. Such small molecules-ligands are promising as novel anticancer drug candidates. Information in huge number of publications would be convenient to systematize using the stability of the complexes formed by the components of the systems in question or by the ability of ligand to bind G4 molecules present in the solution. However, in the present, such systematization is difficult to perform. The lack of reliable, quantitative characteristics for G4-quadruplexes binding properties complicates the development of bioinformatics tools, particularly for the analysis of involvement G4 in the inhibition of telomerase activities in vivo and for the discovery of new anticancer agents. To address this issue, robust binding models are needed to investigate G4-ligand interactions.

The TMPyP4 (5,10,15,20-tetrakis-(1-methyl-4-pyridyl)-21H,23H-porphyrin) is one ligand shown to be capable of binding to the G4-structures. The interaction between TMPyP4 and G4 has been extensively studied since it can inhibit the activity of telomerase upon binding to human telomeric sequences [5]. However, its binding mode is still controversial [6]. This cationic porphyrin contains a large π-planar structure and cationic functional groups providing strong π-π stacking and electrostatic interactions with G-quartets and phosphate groups on the G4 DNA. The variations in the UV-Vis absorption and fluorescence spectra of the binding TMPyP4 ligand are normally the result of a change in the molecular conformation of the ligand in addition to the change in the environmental polarity and strong transition moment dipole coupling between the base pairs of DNA and the ligand. The cationic porphyrin G4 interactions have been studied extensively using a wide variety of spectroscopic techniques and isothermal titration calorimetry (several examples reported previously are summarized in Table S1; see Table S1 in the Supplementary Material available online...
The thermodynamic effect of cooperativity may be due to changes in the macromolecule's conformation or redistribution of the electron density at the site due to the influence through oligomers bonds or by the MI between bound ligands via space [17]. A variety of methods have been developed for computational explanation of cooperative interactions of a ligand with set of binding sites [18]. If these sites are equivalent and independent, relevant experimental data may be analysed toward the thermodynamic binding constant and the number of binding sites in terms of a linear Scatchard plot [19]. Since this presumes a most simple binding model, such an approach is susceptible to substantial misinterpretations in practice [20].

The McGhee and von Hippel model which takes into account the MI between nearest neighbouring bound ligands has been the most widely used [21] in combination with the Scatchard equation for description of small molecules with homogeneous polymers. Sometimes those equations are incorrectly used to describe interactions with G4 and other DNA nonhomogeneous oligomers even though the authors of the model have concluded that the binding of ligands known to have heterogeneous binding sites on the DNA cannot be adequately treated based on the conditional probability approach [21]. Although Scatchard analysis is very much attractive owing to pictorial rendition, it has serious implications of unreliability regarding the shape of a Scatchard plot and it is evident that this method is not appropriate for quantitative evaluation of the binding of TMPyP4 with G4. The Job plots analysis of the ligand binding with the G-quadruplex sites accounting for their MI has been described in [12].

To compare the interpretation of spectrometrically measured data using the canonical stepwise model with that of mutually influencing binding sites, we considered the combination of hard model-based fitting and soft model-free Singular Value Decomposition (SVD) [22]. The Rank Annihilation Factor Analysis (RAFA) has also aided in unambiguous revealing of the cooperative interactions. This solution should improve our understanding of the mechanism of the G folding and binding at a molecular level, which in turn will help in highly efficient and selective telomerase inhibitors discovery.

2. Computational Details

2.1. Description of the Model. To simulate G4-ligand interactions the model based upon a multiple set of binding sites has been proposed [12]. When binding with G4 DNA is encountered, then each monomeric ligand is assumed to be bound to one of the sites on the DNA oligomer. The cooperativity is the enhancing of binding affinity in comparison with the sole binding, but anticooperativity is the reduction of binding affinity. The secondary interaction is an important source of ligand binding cooperativity. In DNA-ligand complexes, the arrangement of the ligands decides the total stability of the complex and elucidates that secondary interactions between neighbouring ligands can contribute additional stabilization [23]. To find a sign of cooperative interactions just the influencing of ligand on another ligand
bound in the close vicinity has been taken into account. Consider intrinsic constants which govern the binding of ligand to a set of sites. In general case, the binding of \( N \) ligands among \( N \) vacancies gives \( 2^N \) possible variants of coordination. The one-dimension matrix of site-specific equilibrium constants \( K_{1..N} \) \((K_{1..N} \) are constants for a ligand binding to each isolated site \( 1 \cdots N \)\) and matrix \( \Omega \) of the MI corrections (cooperativity parameters \([\omega_1 \omega_2 \cdots \omega_N]\)) are constructed in accordance with hypothesis about structure of adducts formed in DNA oligomer saturation by a ligand. The values of \( K_{1..N} \) and \( \omega_i \) could be found simultaneously as was described previously [24].

We will start here with looking at the simplest of situations for ligand binding, namely, the interaction with bidentate oligomer. The matrix of configurations \( \mathbf{M} \) has been introduced in order to describe the detailed set of configurations adopted by the macromolecule in microstates. Each line of the matrix \( \mathbf{M} \) displays a microstates configuration through a sequence of zeros in those positions where the sites of multidentate macromolecule are not occupied by a ligand, and with a sequence of ones in those positions where the sites are bound to the ligand. In the course of the macromolecule sites saturation, previously bound ligands can affect the binding of the following ligands. For the oligomer containing two nonidentical binding sites, the matrix \( \mathbf{M}^* \) can be written as

\[
\mathbf{M}^* = \begin{bmatrix}
0 & 0 \\
K_1 & 0 \\
K_1 \omega & K_2 \omega
\end{bmatrix}.
\]  

The product of the nonzero elements in \( \mathbf{M}^* \) provides the column of stability constants of microscopical species

\[
\mathbf{B} = \prod_{j=1}^{N} (\mathbf{M}^* - \mathbf{M} + \mathbf{E});
\]

where the matrix \( \mathbf{E} \) is all ones and the same size as \( \mathbf{M} \). The estimates of the true nonlinear variables \( K_{1..2} \) and \( \omega \) allow calculating the matrix \( \mathbf{C}_{\text{DNA}} \) of DNA oligomer species of concentration for the \( i \)th total concentration of ligand and DNA, correspondingly \( C_L \) and \( C_D \).

\[
\mathbf{C}_{\text{DNA}} = \begin{bmatrix}
[D\text{NA}]_1 & [D\text{NA}']_1 & [D\text{NA}'']_1 & [D\text{NA}']_1 \\
\cdots & \cdots & \cdots & \cdots \\
[D\text{NA}]_{Np} & [D\text{NA}']_{Np} & [D\text{NA}'']_{Np} & [D\text{NA}']_{Np}
\end{bmatrix}
\]  

Here \([D\text{NA}'] = C_D(1 + [L]^2\mathbf{B})^{-1} \) is an equilibrium concentration of free DNA oligomer and \([D\text{NA}''] = [D\text{NA}] [L]^2 \mathbf{B}^T \) are G4 DNA species equilibrium concentrations; \([L] \) is an equilibrium concentration of free ligand; \( S = 1, 2 \) is a stoichiometric coefficient; \( Np \) is the number of experimental points. In our case the sum of microforms equilibrium concentrations \([D\text{NA}']\) and \([D\text{NA}'']\) gives the concentration of oligomer species corresponding to single bound ligand. This species in conventional stepwise complex formation model is regarded as the 1:1 complex. Once we know \( C_{\text{DNA}} \), we can calculate concentrations of free sites and these bound with a ligand.

\[
C_{\text{MonL}} = [C_{\text{DNA}} [E - \mathbf{M}] C_{\text{DNA}} \mathbf{M}] .
\]  

2.2. The Validation of the Proposed Model. According to Lambert–Beer’s law, a spectrometrically measured data matrix \( \mathbf{A} \) is the product of a concentration matrix \( \mathbf{C} \) and a matrix \( \mathbf{S} \) of molar absorptivities of species that contribute to the absorption of light at any wavelength (species pure spectra). The corresponding general matrix equation is given as

\[
\mathbf{A} = \mathbf{CS} + \mathbf{r};
\]

here \( \mathbf{r} \) is a matrix of residuals. The number of rows in matrix \( \mathbf{A} \) is equal to the number of studied solutions with different components concentrations. The number of columns in matrix \( \mathbf{A} \) is equal to the number of wavelengths. To simulate the data matrix \( \mathbf{A} \) a concentration matrix \( \mathbf{C} = C_{\text{MonL}} \) has been computed in accordance with equilibrium model of two-step ligand binding. The total light absorbance is due to the absorption of the occupied \( \text{Mon}_1 L, \text{Mon}_2 L \) and unoccupied sites \( \text{Mon}_1, \text{Mon}_2 \) of oligomer molecules and absorption of free and coordinated ligand \( L \). The data set was designed to simulate an interaction ligand with oligomer (TMPyP4 with G-quadruplex DNA), which has two binding sites. Since the rank \( R \) of data matrix \( \mathbf{A} \) is equal to the rank of \( \mathbf{C} \) or \( \mathbf{S} \), whichever is smaller, and since the rank of either \( \mathbf{C} \) or \( \mathbf{S} \) can be no larger than the total number of spectral species, the rank of \( \mathbf{A} \) can be no larger than the total number of spectral species, provided the number of wavelengths and the number of studied solutions are both greater than the number of spectral species. For simplicity in the current simulations residuals \( \mathbf{r} \) were put to zero. Thus the rank \( R \) is the number of singular values of \( \mathbf{AA}^T \) that are bigger than machine precision \((10^{-10} - 10^{-13})\).

2.3. The Least Square (LS) Fitting Procedure. The fitting procedure should deconvolve the data matrix \( \mathbf{A} \) into matrices of concentrations \( \mathbf{C} \) and molar absorptivities of a matrix \( \mathbf{S} \). Multiplication of both sides of (5) on a pseudoinverse \([22]\) matrix \( \mathbf{C}^+ = (\mathbf{C}^T \mathbf{C})^{-1} \mathbf{C}^T \), computed with an initial estimate of the required parameters, gives a current estimation \( \mathbf{S}^* \) of the true matrix of molar parameters \( \mathbf{S} \). The best set of linear parameters \( \mathbf{S}^* \) for system in question can be calculated explicitly in a linear least-squares calculation by

\[
\mathbf{S}^* = \mathbf{C}^+ \mathbf{A}.
\]

In order to evaluate linear as well as nonlinear parameters for a chemical model such as that described above, a decomposition of the experimental data matrix \( \mathbf{A} \) is executed under a nonnegativity constraint. The product of \( \mathbf{S}^* \) and \( \mathbf{C}^+ \) gives \( \mathbf{A}_{\text{calc}} \) at the current values for nonlinear parameters.
Consequently, the iteration formula for refinement of the required nonlinear parameters can be written as

\[ A_{\text{calc}} = [C^* A]_{0} C^* + \tau, \]  

(7)

where \([\cdots]_{0}\) is the operator that substituted negative values to zero values (nonnegativity constraint only for spectral profiles was applied); \(\tau\) is the residual matrix with unexplained data variance (here \(\tau\) is machine precision). Relevant experimental data should be analysed toward the intrinsic association binding constants \(K_{1,2}\) and intrinsic parameter of cooperativity \(\omega\) to obtain a best fit to the data. When optimal nonlinear parameters were found, the pure species spectra have been calculated in accordance with (6).

The Levenberg–Marquardt nonlinear LS algorithm is used for refinement of parameters [25]. The iterative procedure described above is used to find the minimum of the sum of squares of deviations of all absorbance data matrix elements which were calculated using theoretical model, from the corresponding matrix considered on the current iterative step, calculated in turn by using (7). Refinement was stopped when the relative difference between consecutive iterations fell below a threshold value. The Hamilton factor was used in the present work to compare matrices by applying the following equation:

\[
PE(F_{\text{exp}}, F_{\text{calc}}) = \sqrt{\frac{\text{Trace}\left[(F_{\text{exp}} - F_{\text{calc}})(F_{\text{exp}} - F_{\text{calc}})^T\right]}{\text{Trace}\left[F_{\text{exp}} F_{\text{exp}}^T\right]}} \times 100% ,
\]

(8)

where Trace is the sum of the diagonal elements. Equation (8) was used to calculate lack of fit (lof). In this case, \(F_{\text{exp}} = A_{\text{exp}} ; F_{\text{calc}} = A_{\text{calc}}\). Second, (8) was used to compare the distribution diagrams calculated by application of conventional stepwise chemical model from the ones simulated by the cooperative model. In this case, a concentration matrix \(F_{\text{exp}} = C_{\text{DNAL}}\) was calculated using a model, and a matrix \(F_{\text{calc}} = C_{\text{calc}}\) was calculated in turn by using (3) on the last LS iterative step. In all cases, Matlab-2014® software was used in the calculations.

3. Results and Discussion

3.1. Cooperative Binding (Data Matrix \(A_{1a}\)). The UV-Vis absorption spectra changes of an aqueous solution in binding reactions of TMPyP4 and G4 in all known cases are typically similar. To simulate data set one have to construct bathochromic red shifts 15–20 nm and hypochromicities at \(\lambda_{\text{max}}\) (60–70%) of the Soret band were observed during coordination TMPyP4 to G4. Spectra \(S_L\) and \(S_{\text{Mon}}\) used to build the data sets \(A_{\text{exp}}\) are shown in Supplementary Material (Figure S1). In the simplest case, we assume that spectrum \(S_{\text{MonL}}\) is distorted superposition of \(S_L\) and \(S_{\text{Mon}}\). This situation is a binding without spectral interference. More general case of spectral interference (SI) is the mutual perturbation of the ligand spectra bound in the vicinity.

Let us consider the model defined by equilibrium constants \(\lg K_{1,2} = [6.5 \ 6.5]\) and \(\omega = 2.0\), governing the formation of a \(\text{Mon}_L\) species (two equal sites plus a positive binding cooperativity between sites). Simulated data matrix (see Supplementary Material, Figure S2) \(A_{\text{exp}}\) should be written as

\[ A_{\text{exp}} = [L] S_L + C_{\text{MonL}} S_M + \tau. \]

(9)

Here elements of the matrix \(\tau\) are put to zero; \(S_L\) is molar absorptivities of free ligand. Given that two sites are equivalent and have no SI the matrix of molar absorptivities of free and occupied sites I-II is \(S_M = [S_{\text{Mon1}} S_{\text{Mon2}} S_{\text{MonL}} S_{\text{Mon1L}} S_{\text{Mon2L}} S_{\text{Mon12L}}]^T\). The concentration matrix used to calculate the absorbance data matrix is

\[ S_{\text{MonL}} = [[\text{Mon}_1] \ [\text{Mon}_2] \ [\text{Mon}_1L] + [\text{Mon}_1L2] \ [\text{Mon}_2L] + [\text{Mon}_2L2]]. \]

(10)

SVD applied for \(A_{1a}\) yields \(R = 3\) indicating the three optically active chemical species in the full spectrum and local rang \(R = 2\) indicating the species in spectral window of TMPyP4.

To fit the data, in accordance with proposed approach, the conventional noncooperative model of stepwise complex formation first has been tested.

\[ \text{G4} + \text{TMPyP4} \longleftrightarrow \text{G4 (TMPyP4)} \]

\[ \text{G4 (TMPyP4) + TMPyP4} \longleftrightarrow \text{G4 (TMPyP4)}_2 \]

(11)

Here stepwise equilibrium constants \(x(1) = \lg K_1\) and \(x(2) = \lg K_2\) are independent variables and \(\omega = \text{constant} = 1\). However the LS fitting gives a poor fit to the target matrix \(A_{\text{exp}}\). The reproduced \(A_{1a}\) matrix is computed as

\[ A_{\text{calc}} = [L] S_L + C_{\text{DNAL}} S_F + \tau. \]

(12)

Here \(S_F = [S_{\text{DNA}} S_{\text{DNAL}} S_{\text{DNAL}}]^T\); \(\tau\) is lof. Once we analysed simulated data matrix \(A_{1a}\) using a model of independent binding sites, taking into account the existence of 2 sites of 2 types, the best fit was model with two equivalent binding sites \(K_{1,2} = [7.04 \ 7.04]\), and a lof is 0.2%.

Figure 1(a) shows distribution diagrams of hypothetical DNA oligomer between species DNA, DNAL, and DNAL2. As can be seen from Figure 1(a), the resolved distribution of the species significantly differs from the theoretical one (PE 18.4%). Figure 1(b) shows simulated and resolved pure species
spectra. The shape of spectral profiles is due to calculated monomers spectra. The spectrum of species DNA1 2 is doubled spectrum of DNA, 2S DNA 2 = S DNA 2.

RAFA of the Data Matrix A 1a. Given that spectra S DNA and S L are easily measured experimentally, we can calculate the matrix A’.

\[ A’ = A_{exp} - [L] S_L - [DNA] S_{DNA} + \tau. \]  

(13)

With the result of (13), A exp can be divided into two parts, where the known species L and DNA only contribute to the one part. The other part A’ contains linear combination of the unknown spectra. The SVD of matrix A 1a indicated no rank annihilation of A 1a including concentration window of the ligand. This result conforms that spectral species are inaccurately recognized.

The difference between theoretical and calculated fractions of free sites and these bound with a ligand, that is, Mon1, Mon2, Mon1L, Mon2L, MonL2, also can be seen. However, the concentration of free monomer is calculated correctly. The matrix A” may be written in the form:

\[ A” = A_{exp} - [L] S_L - ([Mon1] [Mon2]) [S_{Mon1} S_{Mon2}^T] + \tau. \]  

(14)

The reduced data matrix A” has been obtained by subtracting the contribution of the proposed model species from the complete data set. The rank of the matrix A” is less than the rank of the full A 1a matrix R” = R – 1. So in the full spectral range one species is correctly recognized.

The rank annihilation completes the conclusion that sites are indistinguishable monomers.

3.2. Anticooperative Binding without SI (Data Matrix A 1b). To simulate a data matrix A 1b obeying (9), the same spectra as in the previous example were used (see Figure S1). Concentrations are calculated from the model \( \lg K_{1,2} = [6.5 \ 6.5], \omega = 0.3 \). Fitting the absorbance data matrix using conventional stepwise model without cooperativity gives the fit (A 1b) = 0.0%. Here the best fit gives model considering 2 sites of 2 different types \( \lg K_{1,2} = [6.79 \ 5.16] \). The stepwise model provides a good fit to simulated data. Consequently, we have a model ambiguity as a result of the rotational ambiguity. All species fractions quantified very well (see Table 1).

RAFA of the Data Matrix A 1b. As can be seen from Table 1, SVD is applied on A 1b, A 1b, and A 1b giving the different result as for the above case of positive cooperativity. One can see the rank annihilation in full spectral range and in a spectral window of ligand. This result suggests that subtracted spectral species are correctly recognized. The rank of the matrix A” is less than the rank of the full A” matrix R” = R” – 1. So in the full spectral range extra one species is correctly recognized.

3.3. Cooperative Binding with SI (Data Matrix A 1c). In the previous examples, we have used an assumption that all G4-ligand complexes formed possess the same absorbance spectrum, irrespective of the binding mode. However this assumption is violated in most actual systems, probably in each system with multiple binding sites. We will use SI when binding in each site leading to a change in the spectrum of ligand in the vicinity. Given that bound ligands have SI, an experimental matrix A 1c should obey (12) where the matrix of molar absorptivities is
Table 1: Intrinsic binding constants calculated for simulated ligand G4 DNA equilibrium systems.

| Data matrix | \[\text{lg}K_1, \omega\] | \(\text{lg}K_{1-2}, \omega\) | Parameters | Id. sites | Cooperative binding without SI | Anticooperative binding without SI | Cooperative binding with SI | Anticooperative binding with SI |
|-------------|----------------|----------------|------------|----------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| \(A_{1a}\) | \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\) | \([7.04, 7.04]\), \([7.04, 7.04]\), \([7.04, 7.04]\), \([7.04, 7.04]\) | \(0.2, 10.0\) | \(1.0, 10^*\) | \(18.4, 10^*\) | \(10.4, 10^*\) | \(2.0, 10^*\) | \(1.0, 10^*\) |
| \(A_{1b}\) | \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\) | \([6.79, 5.16]\), \([6.79, 5.16]\), \([6.79, 5.16]\), \([6.79, 5.16]\) | \(0.3, 0.8\) | \(0.3, 0.8\) | \(19.4, 2.3\) | \(10.0, 10^*\) | \(1.0, 10^*\) | \(1.0, 10^*\) |
| \(A_{1c}\) | \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\) | \([6.75, 6.75]\), \([6.75, 6.75]\), \([6.75, 6.75]\), \([6.75, 6.75]\) | \(1.0, 1.0\) | \(0.8, 19.4\) | \(2.3, 10.0\) | \(10.0, 10^*\) | \(1.0, 10^*\) | \(1.0, 10^*\) |
| \(A_{1d}\) | \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\), \([6.5, 6.5]\) | \([6.79, 5.16]\), \([6.79, 5.16]\), \([6.79, 5.16]\), \([6.79, 5.16]\) | \(1.0, 1.0\) | \(1.0, 1.0\) | \(4(3), 3(3)\) | \(4(3), 3(3)\) | \(4(3), 3(3)\) | \(4(3), 3(3)\) |

*Parameter is fixed; **\(R < 10^{-4}\) ≈ 0; \(C_L + C_P = 5\mu\text{M}\).
is concentration matrix calculated from the before used binding model \( \lg K_{1,2} = [6.5\ 6.5] \), \( \omega = 2.0 \). The distribution plot of resolved oligomer species is very similar to these shown on Figure 1. SVD is applied on \( A_{1,2} \) yielding four main factors defining absorbance variance \( (R = 4) \), and the intrinsic absorption of the ligand is determined by three factors \( (R_L = 3) \). With the application of (13), the rank annihilation was not achieved. This suggests that all forms are calculated with poor accuracy. Application of (14) for total spectral range gives \( R'' = 3 \) which means the correct calculation of only the monomer contribution.

### 3.4. Anticooperative Binding with SI (Data Matrix \( A_{1d} \)).
Consider the binding model \( \lg K_{1,2} = [6.5\ 6.5] \), \( \omega = 0.3 \). The model is used to simulate anticooperative binding with SI. In this example, the model ambiguity is the result of LS fitting. The presence of SI does not affect the shape of oligomer distribution diagram shown on Figure 2. However, the result of RAPA is not the same. The identification of sites identity is ambiguous in this case. As can be seen from Table 1, the result of SVD application on matrices \( A_{1d}, A_{1,2}^t \), and \( A_{1d}'' \) is the same as for these for two types of sites with different spectral characteristics (see below).

### 3.5. The Binding to G4 DNA with Different Sites (Data Matrix \( A_{3e} \)).
Consider lastly an oligomer which has different binding sites. Consequently each site should have different affinities for the ligand and may have a different absorbance signal. The model with a strong binding site and weaker binding site \( \lg K_{1,2} = [7\ 6] \) to simulate the data matrix was used. Simulated data can be decomposed by the model of two equivalent binding sites and anticooperativity \( \lg K_{1,2} = [6.74\ 6.74] \), \( \omega = 0.58 \) with excellent precision (see Table 1). In this case to simulate data use (12), but reproduced data matrix is calculated in accordance with (9). Resolved distribution of G4 oligomer species well reproduce those used for simulation. The rank of a data matrix constructed in accordance with (12) is equal to the rank of \( C (R = 4) \). The rank of both \( A_{2a} \) and \( A_{3e} \) in spectral window \( (R_L = 3) \) is less than the number of spectral species in full spectral range because the light absorption by monomer species is zero in this interval. Nevertheless, the rank of \( A'' \) along with \( A'' \) two units less compared with \( A \) can be seen due to rotational ambiguity. Moreover we can see the same sequences of \( R (R_L), R'' (R_L') \), and \( R'' (R_L'') \) regardless whether or not there is spectral MI. Thus, given example of binding shows inapplicability of RAPA to indicate the fact that sites are different or the same sites bind in anticooperative fashion.

### 3.6. The TMPyP4 Binding with Nucleotide Sequence 5’-(CG3)2CGC(AG3)2G-3’ Experimental Data (Matrix \( A_{3e} \)).
To illustrate the applicability of the suggested approach to model porphyrin binding with a macromolecule, a porphyrin TMPyP4 binding to 5’-(CG3)2CGC(AG3)2G-3’ was selected as experimental example. UV-absorbance experimental data published previously [15] has been used for further numerical treatment. In the original work [15] mole-ratio experiments to study the G4-ligand interaction were performed. When solution of G4 is added to solution of TMPyP4 in buffer pH 7.1, at 25°C the induced bathochromic red shifts and hypochromicities for the Soret band clearly indicate the DNA-ligand complexation. A stepwise complex formation model has been applied in [15] to interpret the experimental data (matrix \( A_{3e} \)). Conventional stepwise complex formation algorithm best fits the data matrix which included one strong binding site and one weak binding site \( (\lg K_{1,2} = [7.07\ 5.79]) \), \( \text{lof}(A_{3e}) = 0.25\% \). However, the same fit gives a model with two equivalent anticooperative binding sites. The calculated intrinsic binding constants \( K_{1,2} \) and the cooperativity parameter \( \omega \) are summarized in Table 2. The calculated distribution diagrams for this mole-ratio spectrometric titration of G4 by TMPyP4 for both models are compared in Figure 2. For this G4 DNA example of binding interaction, the LS fitting discloses a model ambiguity. The values of \( R'' (R_L'') \) we have given in Table 2 equal the values of all these quantities which are given in Table 1 for anticooperative binding with identical sites without SI. Based on this observation, one can suggest a model of anticooperative assembling of TMPyP4 and G4 DNA with identical binding sites without SI. In addition, the calculated pure spectra for species involved in the equilibrium are shown in Figure 2. The spectra are calculated according to the following equation:

\[
S = A_{3e}'' \left[ [\text{Mon}_L] + [\text{Mon}_L] [\text{Mon}_L,2] \right]''
\]

\[
= A_{3e}' \left[ [\text{Mon}_L] + [\text{Mon}_L] [\text{Mon}_L] + [\text{Mon}_L,2] \right]'.
\]
and the mutual influence between bound ligands, which is essential to G4 DNA in vivo function annotation, can be done for sites involved in cooperative ligand binding. Unfortunately, the discovery of the sites identity is hindered when sites are involved in anticooperative binding. It is shown that the spectrometrically measured titration curves can be described ambiguously using the model of two different and independent binding sites as well as that of two mutually influencing identical sites in the case of anticooperative binding.

**Conflicts of Interest**

The author declares that they have no conflicts of interest.

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