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Comparative Analysis of 1,9-Dimethyl-Methylene Blue and Toluidine Blue Interactions with ct-DNA, G-Quadruplex DNA, and ssDNA

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ABSTRACT

This study presents a comprehensive investigation into the interactions of two distinct synthetic dyes—1,9-Dimethyl-Methylene Blue and Toluidine Blue —with different DNA structures, namely calf thymus DNA (ct-DNA), G-quadruplex DNA, and single stranded DNA (ssDNA). The objective of this research is to elucidate the molecular affinities of these dyes for specific DNA structures and explore their potential applications in molecular biology and diagnostics. The experimental approach involved detailed UV-visible spectroscopic analyses, to probe the binding affinities of each dye with ct-DNA, G-quadruplex DNA, and ssDNA. The study aimed to assess the selectivity of these dyes towards the unique structural features of each DNA entity. The binding stoichiometry is defined from Job's method. The selectivity of the dyes towards DNA also investigated with competitive dialysis experiments. The binding stoichiometry were 1:1 for 1,9-Dimethyl-Methylene Blue and Toluidine Blue and G-quadruplex DNA or ssDNA. Besides, results indicate that 1,9-Dimethyl-Methylene Blue and Toluidine Blue exhibit a pronounced affinity for G-quadruplex DNA, and ct-DNA. While single-stranded DNA is a fundamental component of DNA replication and transcription, our dyes exhibit lower affinity for this structure. The selectivity is advantageous, as it allows for the discrimination between single-stranded and structured DNA regions. The potential applications in studying DNA dynamics and unwinding processes are vast.

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Introduction

Cancer is a complex disease that develops with the accumulation of DNA damage and changes in genome structure and function (Davalos and Esteller, 2023). The cell proliferation increases in cancerous cells. Uncontrolled cell division is one of the most prominent findings in cancer. Due to increased metabolism, DNA replication and transcription are increased in the cancerous cell.

G-quadruplex DNAs are DNA structures that occur in guanine-rich regions of the genome. They have emerged as attractive targets for therapeutic intervention due to their association with critical biological processes, including gene regulation and telomere maintenance (Hirt et al., 2014). These secondary structures are formed by stacking G-tetrads on top of each other. G-tetrads are formed by connecting four guanine bases via Hoogsteen hydrogen bonds. G-quadruplex DNAs become stable with the presence of cations such as sodium or potassium. Stabilization of these structures is important. Because the stabilization of these has been associated with a reduction of telomere ends or a lower oncogene expression (Zegers et al., 2023). In addition, G-quadruplex DNA stabilizing or perturbing ligands have very important potential for use in imaging, theranostic and diagnosis (Holden et al., 2023). Therefore, G-quadruplex DNA is a very important target in the treatment of cancer (Figueiredo et al., 2023).

Small molecules that specifically interact with G-quadruplex structures can modulate their stability and function (Awadasseid et al., 2021). These molecules often possess aromatic moieties and functional groups that facilitate binding to the guanine tetrads (O'Hagan et al., 2019; Pirota et al., 2020). The recognition of G-quadruplexes by small molecules is a highly specific process, guided by factors such as shape complementarity, electrostatic interactions, and hydrogen bonding. The consequences of G-quadruplex DNA-small molecule interactions extend beyond structural stability (Biver et al., 2022; Mendes et al., 2022; Kaushik et al., 2011; Du et al., 2010). These interactions can influence gene expression,

telomere maintenance, and DNA replication. Small molecules that stabilize G-quadruplex structures may act as potential anticancer agents by interfering with telomerase activity or modulating the expression of oncogenes (Takahashi et al., 2021; Lerner and Sale, 2019; Salvati et al., 2007). Ligands with selective affinities hold immense potential for applications in biotechnology and diagnostics (Yang et al., 2010). Their ability to discriminate between various DNA structures makes them valuable tools for designing DNA sensors, detecting specific sequences or structural motifs associated with diseases, and advancing our understanding of molecular interactions within living systems (Li et al., 2010).

Numerous human malignancies, including as osteosarcomas, lymphomas, leukemias, cervical, lung, breast, and prostate cancers, are associated with overexpression of the c-MYC proto-oncogene. High c-MYC expression levels are also frequently linked to a poor outcome for therapy. A variety of processes, including as gene amplification, translocation, and straightforward transcriptional upregulation, can result in c-MYC overexpression (Mathad et al., 2011). Due to these properties, c-MYC234 G-quadruplex structure belonging to the c-MYC promoter region was studied.

1,9-Dimethyl-Methylene Blue and Toluidine Blue are both synthetic dyes that belong to the thiazine dye family. These two dyes are known to interact with DNA. The structural differences contribute to variations in their interactions with DNA. In this study, the interactions of these dyes with ssDNA, G-quadruplex DNA and ct-DNA were examined to understand whether there was a difference in the affinities of these dyes to DNA structures.

Materials and Methods

Double distilled water (dd-H_2O) was used in the preparation of main stock solutions throughout all experiments. pH 7.4 TRIS-HCl buffer solution containing 100 mM KCl was used for dilutions. pH adjustment of the buffer solution was made with a Sartorious basic model pH meter. ct-DNA (Sigma-Aldrich) stock solution was prepared to get a concentration of 1 mg/mL, c-MYC2345 and ssDNA (Alpha DNA, Canada) stock solutions were prepared as 100 micromolar. The solutions were diluted with buffer solution to appropriate concentrations to be used in experiments. c-MYC2345 and ssDNA solutions were incubated at 95ºC for 5 minutes and cooled to room conditions. ct-DNA concentration was calculated from the absorbance value and extinction coefficient of 6600 $M^{-1}cm^{-1}$ (Reichmann et al., 1954).

UV-vis spectra were scanned with a Shimadzu UV 1800 model spectrophotometer and using quartz cuvettes. The stock solutions of 1,9-Dimethyl-Methylene Blue (1,9 DMB) zinc chloride double salt (Sigma-Aldrich) and Toluidine Blue O (TBO) (Sigma-Aldrich) were prepared in dd-H_2O and further diluted with buffer solution. The solution is protected from light.

For the spectrophotometric titration, 8 µM of 1,9 DMB was prepared by diluting the stock solution in pH 7.4 TRIS-HCl buffer solution containing 100 mM KCl. The concentrated DNA (150 µM for ct-DNA, 10 µM for both c-MYC2345 and ssDNA) solution was added in small portions and pipetted several times to mix the solutions homogenously. The absorption spectrum was scanned after each addition and the absorbance values were recorded for each titration point. The control experiment was performed without DNA.

The titrations of TBO were also conducted with the same experimental procedure.

The binding constant, Kb was calculated from (Kocak et al., 2016; Yılmaz et al., 2021):

$$L + DNA \text{ ==== } L\text{-}DNA$$

$$K_b = \frac{[L-DNA]}{[L][DNA]}$$

$$[L]_t = [L] + [L-DNA]$$

Then

$$K_b = \frac{[L-DNA]}{([L]_t - [L-DNA])[DNA]}$$

$$K_b[L-DNA]^2 - \left(1 + K_b[L]_t + \frac{K_b[DNA]}{s}\right)[L-DNA] + \frac{K_b[L]_t[DNA]}{s} = 0$$

$[L\text{-}DNA]/[L]_t$ is the bound fraction and calculated from the absorbance data:

$$A_a = A_f + A_b$$

Where A_a, A_f and A_b is the apparent, free and bound absorption.

$$\varepsilon_a[L]_t = \varepsilon_f[L] + \varepsilon_b[L-DNA] = \varepsilon_f([L]_t - [L-DNA]) + \varepsilon_b[L-DNA]$$

Then

$$\frac{[L-DNA]}{[L]_t} = \frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f}$$

$$\frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{b - \sqrt{(b^2 - \frac{4K_b^2[L]_t[DNA]}{s}}}{2K_b[L]_t}$$

$$b = 1 + K_b[L]_t + \frac{K_b[DNA]}{s}$$

Where εf, εb, and εa are the extinction constants for free, bound dye and apparent mixture of dye, DNA and dye-DNA when both Dye-DNA complex and either one of the dye or DNA is freely available. The Solver tool of excel software (Microsoft) was used for the nonlinear equation solution.

The stoichiometry of binding was determined with Job's method. A series of solutions were prepared where the total concentration (dye+DNA) remains constant, and only the mole fraction of one of the components varies systematically. The absorbance of the solutions was measured as the mole fraction of one component changes. The absorbance against the mole fraction of the dye was plotted.

Table 1. The sequences for c-MYC2345 and ssDNA

c-MYC2345	5'- TGA GGG TGG GGA GGG TGG GGA A
ssDNA	5'- TTC CCC ACC CTC CCC ACC CCT CA

(a) (b)

Figure 1. Chemical strüktürel of a) 1,9-Dimethyl-Methylene Blue and b) Toluidine Blue O.

Competitive Dialysis

A semi-permeable dialyses membrane with a MWCO 3500 (SERVA, Membra-cel, dialysis tubing) that is appropriate for the molecules under investigation was used. 1000 µL of 75 µM of each DNA sample were placed into dialyses membrane. All membranes were placed into a baker which contains 400 mL 1 µM of dye solution. The membrane was allowed for the selective diffusion of the dye molecules while maintaining the competitive aspect of the dialysis. The concentrations of the dyes after dialysis (24 h) was measured spectrophotometrically to assess the competitive interactions (Guittat et al., 2003).

Results and Discussion

UV-Visible Spectrophotometric Titration

UV-Visible spectrophotometric titration is a versatile technique used to investigate molecular interactions by monitoring changes in absorbance as a function of a titrant's concentration. In the context of studying G-quadruplex DNA, this technique allows researchers to observe alterations in the electronic structure of the DNA and the small molecule ligand (Feizi-Dehnayebi et al., 2021). The UV-Visible spectrophotometric titration of small molecules with G-quadruplex DNA is a powerful technique that provides valuable information (such as binding constants) about the binding interactions between the G-quadruplex DNA and small molecule.

The UV-Visible spectrum of 1,9-Dimethyl-Methylene Blue typically exhibits absorption peaks in the visible region of the electromagnetic spectrum. The positions and intensities of these peaks are indicative of the specific electronic transitions occurring within the molecule. The binding of 1,9 DMB to DNA can induce conformational changes in the DNA structure. This interaction may cause alterations in the stacking and arrangement of DNA base pairs. These structural changes can, in turn, influence the electronic interactions between the dye and the DNA, leading to changes in the electronic spectrum. The electronic spectrum of 1,9 DMB typically includes absorption peaks corresponding to specific electronic transitions. The presence of DNA can shift these peaks, broaden them, or induce new peaks, indicating changes in the electronic states of the dye.

As can be seen from Figure 2 and Figure 3, 1,9 DMB and TBO have a large absorption band between 550-700 nm range. The addition of DNA to dye resulted in hypochromic effect on these band. Besides hypochromic effect, the wavelength shift also observed. Especially for 1,9 DMB, the interaction with ct-DNA and c-MYC2345 resulted in a significant hypsochromic effect.

The binding constant, also known as the association constant (K_b), quantifies the strength of the interaction between two molecules, typically a ligand and a receptor. The K_b values for 1,9 DMB were $(1.09\pm0.02)x10^7$, $(1.67\pm0.18)x10^6$, and $(1.89\pm0.83)x10^7$ M for c-MYC2345, ssDNA and ct-DNA, respectively. As can be seen, the K_b values for c-MYC2345 and ct-DNA were found ten times higher than Kb of ssDNA. The K_b values for TBO were $(5.75\pm4.25)x10^6$, $(2.11\pm1.18)x10^6$, and $(1.82\pm1.46)x10^7$ M for c-MYC2345, ssDNA and ct-DNA, respectively. The affinity of TBO to ct-DNA was found higher.

The determination of binding stoichiometry for small molecule-DNA is important to evaulate the mechanism. The small molecule typically has functional groups that can form specific interactions with the G-quadruplex structure. These interactions may include hydrogen bonding, π-stacking, electrostatic interactions, and van der Waals forces (Chen et al., 2013). The binding stoichiometries of dyes with c-MYC2345, ssDNA were evaluated using Job's method. As shown Figure 4 - Figure 5 the binding stoichiometries were found 1:1 for all circumstances. The mechanism of 1:1 G-quadruplex-small molecule interaction can vary depending on the nature of the small molecule and the specific G-quadruplex structure involved. The 1:1 binding stoichiometry may be due to end stacking. End-stacking refers to the interaction of aromatic moieties at the termini of adjacent G-tetrads in a G-quadruplex. This phenomenon plays a pivotal role in stabilizing the G-quadruplex structure, contributing to its overall stability. The aromatic rings, often derived from guanine bases, engage in π - π stacking interactions, creating a continuous stacking interface at the ends of the G-tetrads. End-stacking interactions not only enhance the thermal stability of the G-quadruplex but also influence its folding topology. It's important to note that the actual mechanism can be highly specific and may involve multiple steps and factors. Additionally, experimental techniques such as NMR spectroscopy, X-ray crystallography, or molecular modeling needed often employed to elucidate the details of the binding mechanism and the structural changes that occur upon interaction.

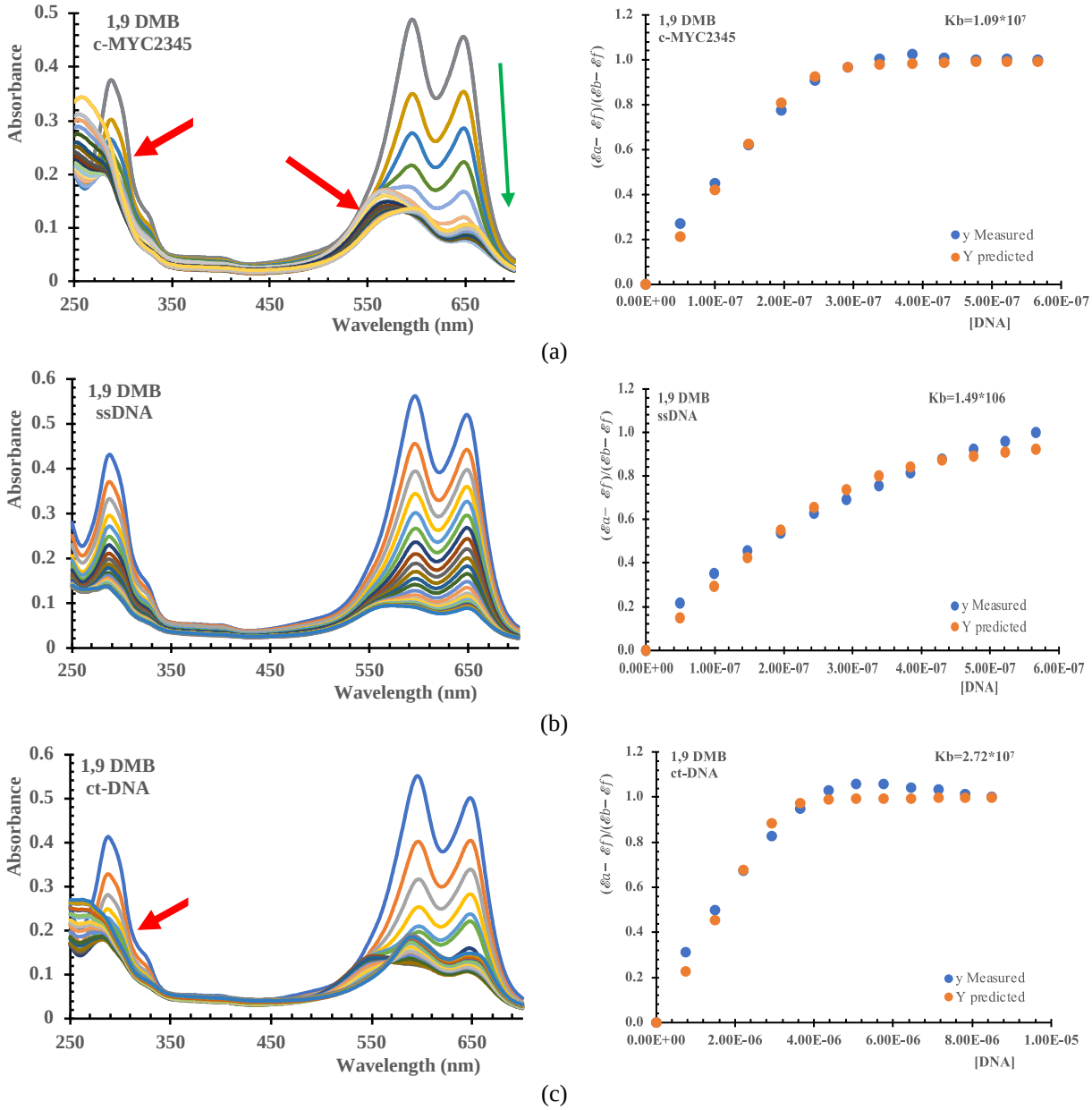

Figure 2. UV-Visible titration spectrum and binding curve for 1,9 DMB (8 µM) with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl, unit of DNA concentration and Kbs are M)

Competitive Dialysis Experiments

In biochemical research, competitive dialysis is used to explore interactions between biomolecules, such as nucleic acids, enzymes, and small molecules (Ren and Chaires, 1999). This aids in unraveling the complex networks of molecular associations within living systems. Competitive dialysis takes the principles of traditional dialysis a step further by introducing a competitive element into the equation. This technique involves the co-dialysis of two or more molecules through a semi-permeable membrane, where they compete for binding sites or interactions. The relative affinities of these molecules for the binding sites on the membrane or for each other can be studied, providing valuable insights into the dynamics of molecular interactions.

Competitive dialysis is a technique employed to study the relative affinities of a molecule, in this case, a dye, for different DNA structures. This method involves setting up a dynamic equilibrium where the dye competes for binding sites on various DNA structures in a controlled environment. The dialysis membrane allows for the exchange of small molecules while retaining the larger DNA structures, enabling the quantification of binding affinities. As the experiment progresses, the concentrations of the dye in each membrane reach an equilibrium based on their respective affinities for the DNA structures. By monitoring the concentrations over time, we can derive information about the relative binding affinities of the dye for single-stranded DNA, G-quadruplex, and calf thymus DNA.

As can be evaluated from Figure 6, the affinities of 1,9 DMB and TBO to c-MYC2345 and ct-DNA are higher. The equilibrium concentration of TBO for c-MYC2345 was 35.28 µM which is the highest value for competitive dialysis experiment.

Conclusion

When all experimental results were evaluated together, it was observed that both dye molecules showed higher affinity for ct-DNA and c-MYC2345 DNA. Although the experiments did not provide information about the binding mechanism, basic information about binding stoichiometry, binding constants and relative affinities was obtained. This study is a basic study in which the interactions are determined, and further studies are needed to determine the binding mechanism and thermodynamic data on binding.

The study of G-quadruplex-small molecule interactions is a dynamic field, and researchers continue to explore and understand the intricacies of these molecular interactions for potential therapeutic applications. While the promise of G-quadruplex DNA-small molecule interactions in drug development is evident, challenges such as cellular uptake and specificity remain. Advances in structural biology techniques, including X-ray crystallography and NMR spectroscopy, have provided invaluable insights into the detailed interactions at the molecular level, aiding in the rational design of small molecules with enhanced binding affinity and selectivity.

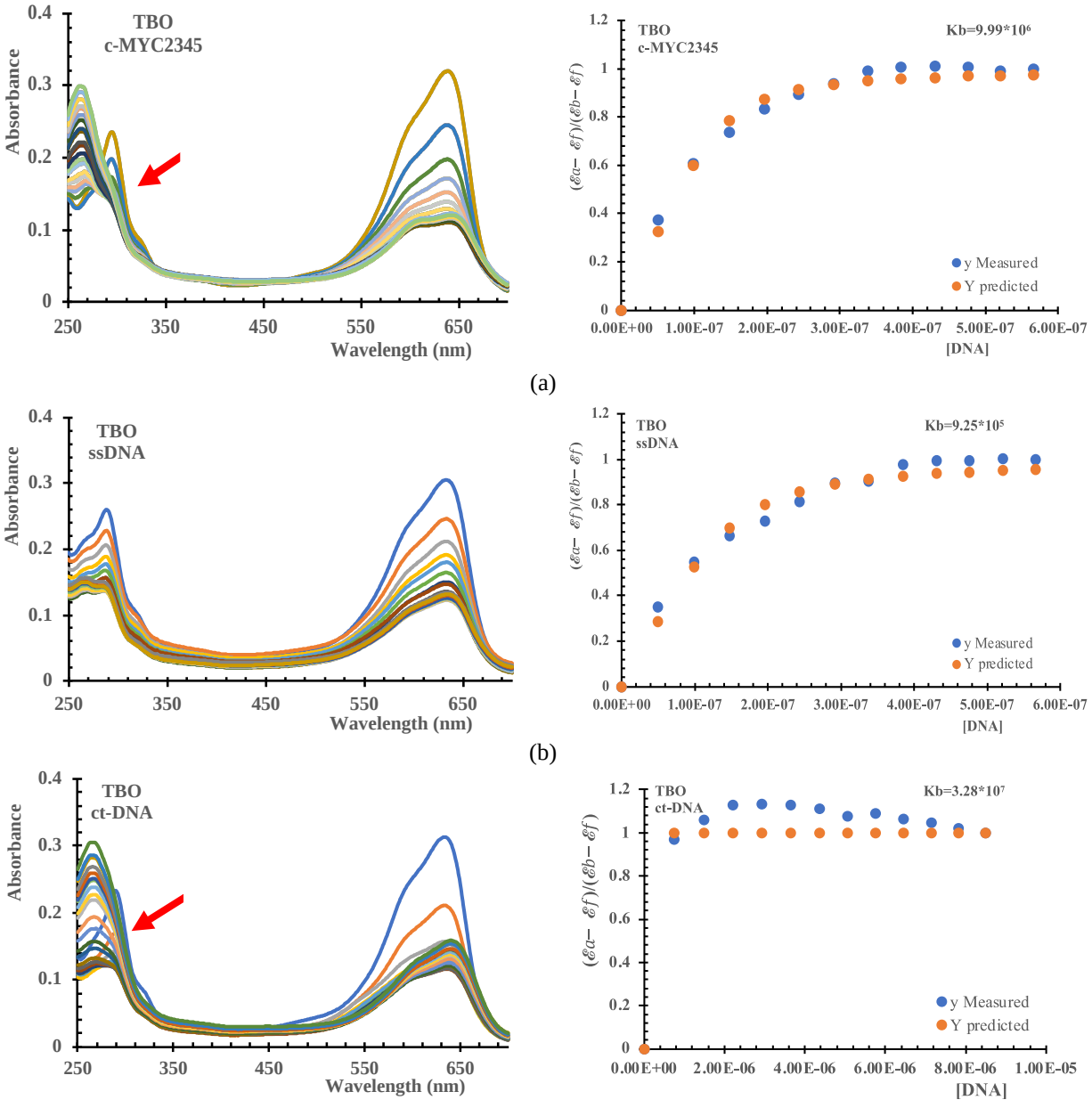

Figure 3. UV-Visible titration spectrum and binding curve for TBO (8 µM) with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl).

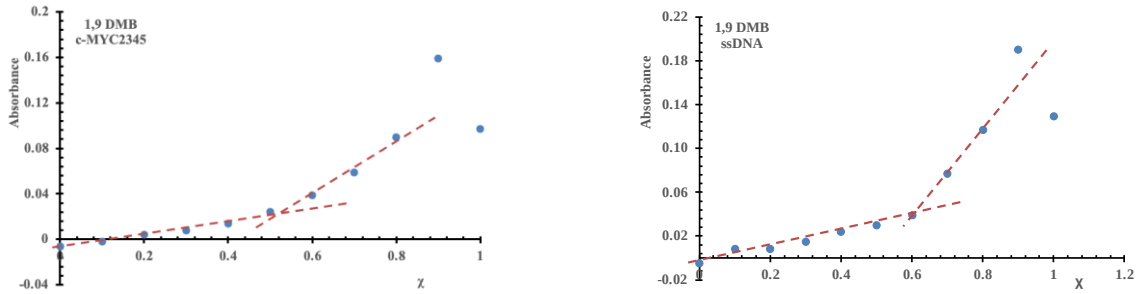

Figure 4. Job's Plot for 1,9 DMB with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl).

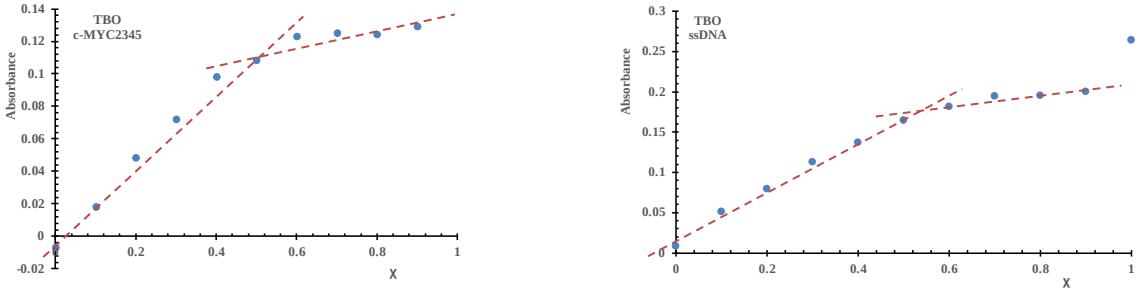

Figure 5. Job's Plot for TBO with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl).

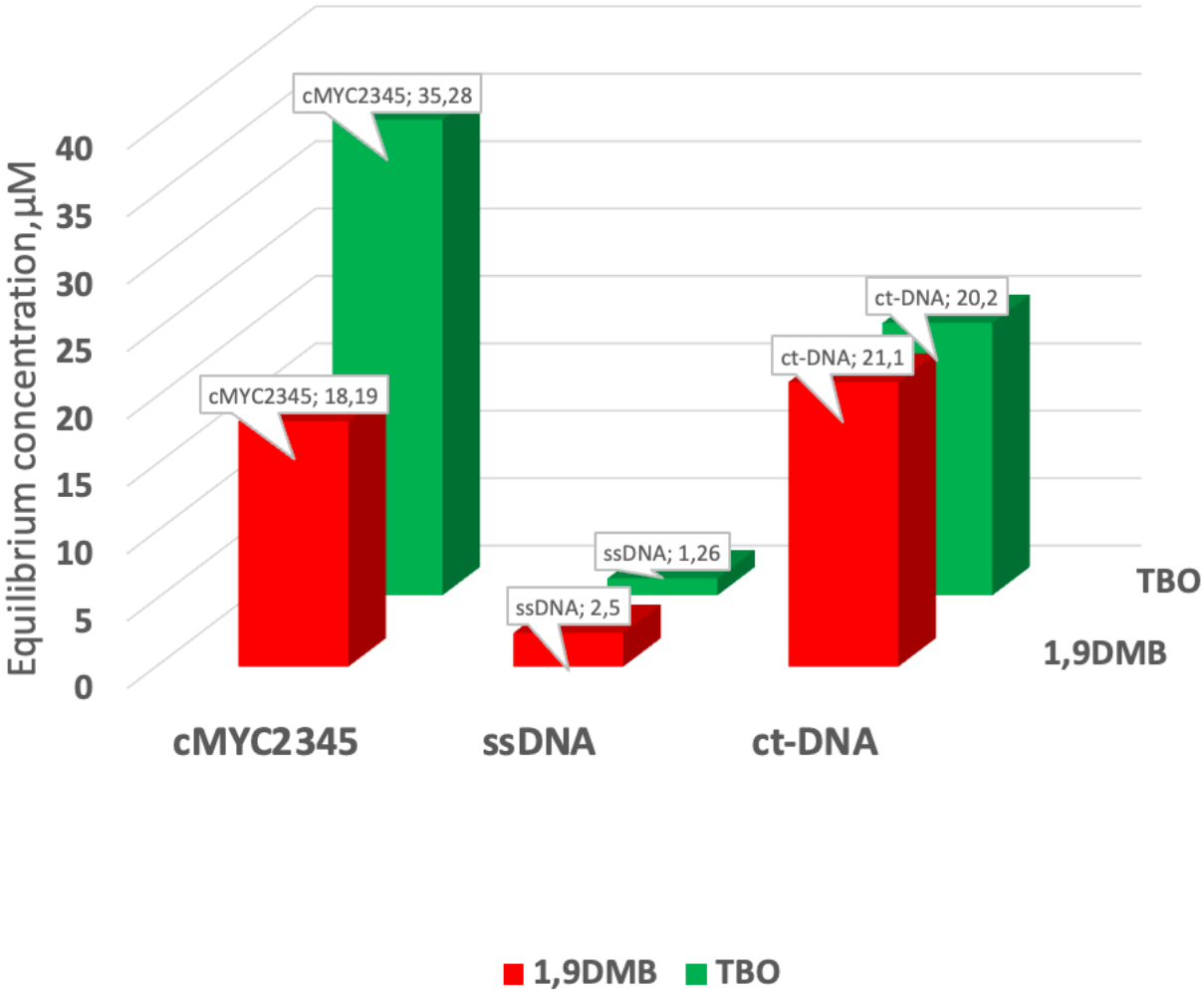

Figure 6. Competitive dialysis

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