Protein-Ligand (Drug) Interactions Using Simplified Equilibrium Dialysis Setup

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***Abstract*-Ultrafiltration, fluorescence spectroscopy, fluorescence-detected competitive binding, X-ray crystallography and chromatographic methods (HPLC) have all been used to evaluate protein-ligand (drug) interactions. Although these methods provide useful binding information, they are constrained by high costs, low sensitivity, and complicated instrumentation. Accurate, fast and simple methods of determining the affinity of a small molecule for a target protein are needed to speed the discovery of new medications and biological probes. A simplified lab-made equilibrium dialysis setup was coupled with absorption spectrophotometry and tested with protein-ligand interactions using methylene blue (MB) and bovine serum albumin (BSA) as models. The results from the absorption spectroscopic analysis showed a reduction in the absorption of methylene blue after dialysis as a result of MB-BSA complex formation with high association constant. The wavelength for maximum absorption (λmax) of methylene blue was found to be 664 nm, and the time for equilibration was approximately 2 hours. The K_d, B_{max} and n were 9.2 µM, 0.0143 µM/min and 0.5051, respectively, and the association constant was $K_a = 1.087 \times 10^5 M^{-1}$. The thermodynamic constants ΔG, ΔH and ΔS were found to be 12.392 kCalmol^{-1}, -13.227 kCalmol^{-1}, and -41.63 Calmol^{-1}K^{-1}, respectively, and the activation energy was 56.23 kCalmol^{-1}K^{-1}. This method is simple and cost effective, and can be used to measure the drug-protein binding parameters with the aid of basic laboratory equipment.**

Keywords- Home-made Equilibrium Dialysis Setup; Methylene Blue; Bovine Serum Albumin; Absorption Spectrophotometry

I. INTRODUCTION

Binding of a small molecule (ligand) to its target macromolecule is an important biochemistry phenomenon. Applications are seen in signal transduction pathways, membrane transport, allosteric regulation, interaction of drugs with receptors, and immobilization of enzymes [1]. The process of binding in drug design is widely used because drug-protein interaction is the basis for the design of most pharmacological compounds. Based on the affinity of a drug to serum proteins, its distribution, metabolism, and efficacy can be altered, and many promising new drugs are rendered ineffective because of their unusually high affinity for their target molecules or circulating serum proteins. It is necessary to determine the unbound (free) fraction of a drug as a means of monitoring its efficacy because only the unbound drug is available for distribution and pharmacological activity. It is becoming increasingly important to understand and characterise ligand/drug-binding interactions for a variety of endogenous and exogenous compounds [2].

Several techniques are currently used to determine the kinetic and thermodynamic constants associated with protein-ligand binding, each with its own strengths and limitations. Some methods for measuring protein–ligand binding include absorption spectroscopy [3], equilibrium dialysis [4-6], ultrafiltration [5], ultrafiltration Raman difference (UFRD) [7], fluorescence spectroscopy [8], and circular dichroism spectroscopy [3]. Most of these methods measure protein-ligand-binding enthalpies and dissociation constants in the nM–µM range. Unfortunately, these methods are constrained by high cost and low sensitivity, and it is time-consuming to set up the instrumentation involved. Equilibrium dialysis is promising and can be applied at lower concentrations (as low as 1 µM), but may require hours or even days to travel through the membrane and equilibration [7].

Despite these drawbacks, equilibrium dialysis has been shown in several experiments to be conducive for measuring binding constants of small organic molecules to major plasma proteins such as albumin. It is also relatively cheap compared to other methods, and offers the opportunity to study low affinity interactions (concentrations as low as 1 µM) that are undetectable using other methods and perform interaction studies without the use of fluorescent or radio-labeled tags. However, most laboratories cannot afford many of the commercial equilibrium dialysis kits, which have restricted research in such key areas to a small number of organizations who have the required instrumentation. Consequently, there is a need to modify existing methods or develop high throughput methods, which are simpler and more accurate to facilitate research.

The present study aimed at developing a simplified and convenient eppendorf tube-beaker equilibrium dialysis (ED) apparatus that could be conveniently used to characterize protein-ligand interactions. For this study, the interacting species were two common laboratory chemicals used in cell and molecular assays: methylene blue (MB) and Bovine serum albumin (BSA). Methylene blue is a cationic planar molecule, which binds easily to most bio-macromolecules [9] and has good spectroscopic and photo physical properties. As can be seen from its structure shown in Fig. 1, MB is a polycyclic and aromatic dye belonging to the thiazine class. Bovine serum albumin is a homologue of human serum albumin with unusual ligand binding properties [10]. MB-BSA binding was chosen because it is a known interaction, which was adopted to validate the simple equilibrium dialysis setup developed in the current study [9-11]. Because of its easy interaction with most

biomacromolecules, especially proteins, synchronous fluorescence techniques for protein determination have been developed on the basis of MB binding to BSA, which further illustrate this proven interaction [9, 11].

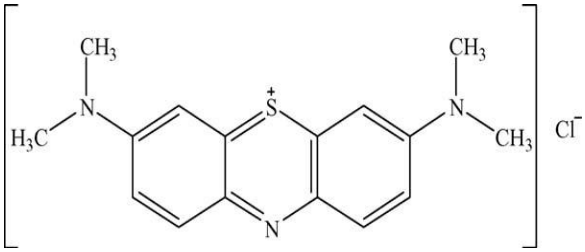


Fig. 1 Structure of methylene blue [10]

The eppendorf-tube-beaker ED apparatus operates on the same principle as any equilibrium dialysis setup with two chambers separated by a semi-permeable membrane that only allows the passage of molecules with a molecular weight less than the molecular weight cut-off (MWCO) of the membrane. Equilibrium is attained between the two chambers, with this approach, which allows for quantitation. The binding parameters of the compounds, including the binding constants (K_a, K_d and n, the number of binding sites on the protein) obtained from binding assays demonstrates the affinity of one ligand for a host molecule, as well as providing insights into understanding the functions of the bound species.

This paper presents a detailed investigation including the kinetics and thermodynamics of the binding of methylene blue to BSA as a model for protein-ligand interaction using a simplified eppendorf-tube-beaker equilibrium dialysis apparatus coupled with UV/Vis spectrophotometry. The results offer a better understanding of the binding mechanisms of methylene blue to serum albumin. Due to the simplicity of the method, it can be easily and inexpensively set up in any basic laboratory to facilitate the study of the binding mechanisms of common therapeutics with proteins.

II. EXPERIMENTS

A. Materials

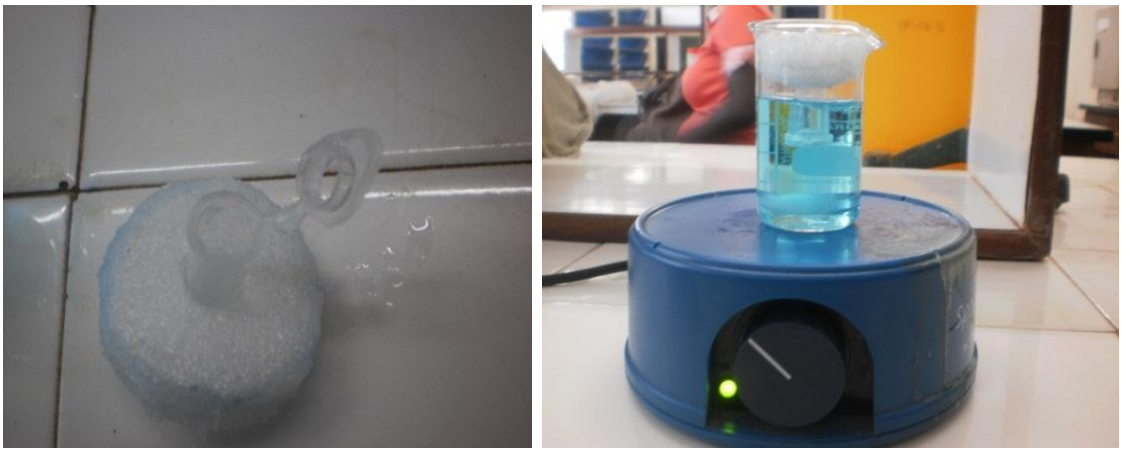
Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA) and methylene blue (MB) from Auro Avenida Exports Pvt Ltd with purities of >99 %. The phosphate buffer components were obtained from May and Baker Ltd. Dagenham, England ($NaH_2PO_4.H_2O$) and BDH Chemicals Ltd. Poole, England (Na_2HPO_4) with purity of 99%.

B. Equipment and Spectral Measurements

The UV–Vis absorbance values were recorded at room temperature on a spectrophotometer (SHIMADZU UV MINI – 1240) equipped with 1.0 cm light path length. Appropriate blanks corresponding to the buffer were subtracted to correct the background absorbances. Sample masses were accurately weighed on Explorer Pro (Ohaus Coorp, Pine Brook NJ USA, made in Switzerland) electronic balance. The pH measurements were taken using pH meter (Kirkhouse Trust Denver Instrument Ultra basic).

C. Equipment and Procedures

The dialysis was made up of two chambers separated by a dialysis membrane. An eppendorf tube was used as the protein chamber for the BSA, and a beaker contained the interacting ligand-methylene blue. The eppendorf tube was perforated at the cap to allow the membrane to access the solutions. Fig. 2 (a) and (b) show the ED setup.



(a) (b)

Fig. 2 Equilibrium dialysis (a) Perforated cap of eppendorf tube (b) Setup for the dialysis

D. Working Conditions

As a preliminary, the wavelength for maximum absorption was confirmed by scanning a 15 µM methylene blue solution from 400 nm to 800 nm. The absorption spectrum of methylene blue is shown in Fig. 3. The stability of the dye solution over the assay period was determined by taking absorbance readings before and after three hours as shown in Fig. 4. The time required to reach equilibrium was also determined. Nine eppendorf tubes were each filled with 1mL of 0.17 µM BSA solution. The opening of each tube was covered with a dialysis membrane and the cap was closed tightly. The tubes were inverted into beakers containing 0.5 µM methylene blue solution and the setups were stirred continuously. The tubes were removed at varying time intervals of 30 mins up to 270 mins and then samples were collected from the methylene blue chamber and analyzed using a spectrophotometer (SHIMADZU UV MINI – 1240). A graph of the MB concentrations against time was plotted as shown in Fig. 5.

E. Effects of Varying Methylene Blue Concentrations on the Binding

The effects of varying methylene blue (ligand) concentration on the binding was determined by dialyzing 20 mL of each of the varying concentrations of methylene blue solutions (0.2 µM – 1.0 µM) against 1 mL of 0.17 µM BSA in an eppendorf tube for 2 hrs. Prior to the dialysis, the initial absorbance values of each concentration of methylene blue were taken. The set-ups were stirred continuously until equilibrium was attained. After 2 hrs, the absorbance of the methylene blue solution in the beaker was measured from which the bound fraction was calculated. The MB-BSA complex for each concentration was plotted against the concentrations, as shown in Fig. 6. Figs. 7 and 8 represent the double reciprocal and scatchard plots, respectively, from the binding data from which the kinetic parameters were estimated.

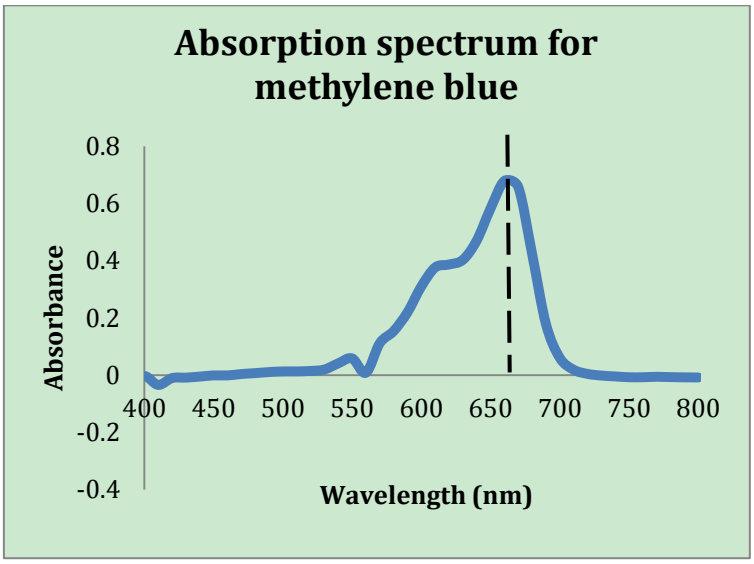


Fig. 3 Absorption spectrum of methylene blue (Absorbance of a 15µM methylene blue solution measured from 400nm to 800nm, the values of which were plotted against various wavelengths to obtain the λmax)

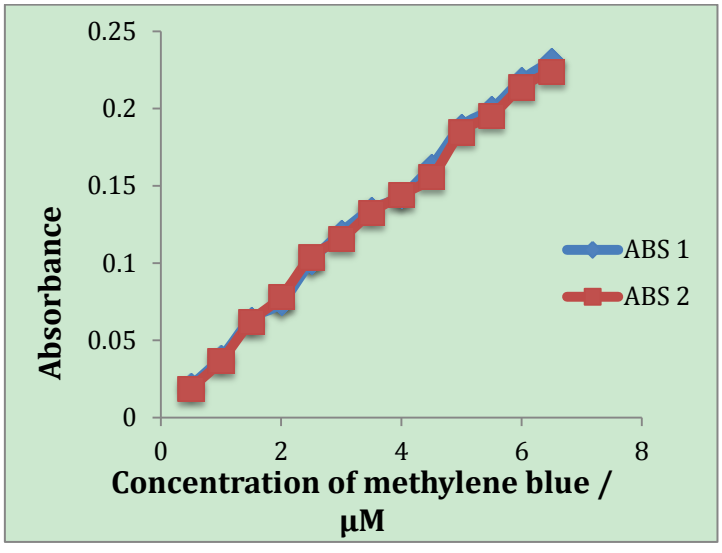


Fig. 4 Stability of methylene blue dye during the dialysis period Absorbance values of varying methylene blue concentrations were taken initially (ABS1) and then again after 3 hours (ABS2) to indicate the stability of the dye)

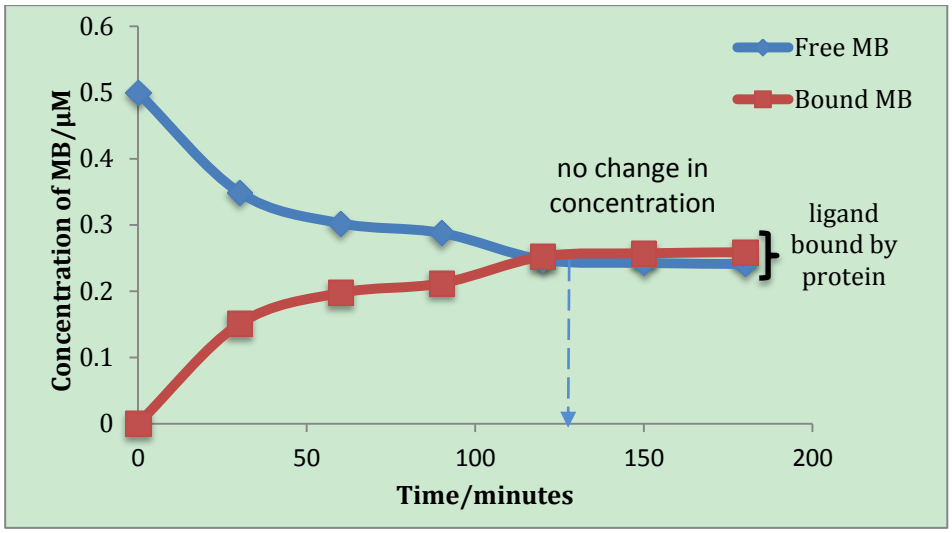


Fig. 5 Change in concentration of bound and free methylene blue with time. An amount of 0.5 µM methylene blue was dialyzed against 0.17 µM BSA at varying time intervals, and the bound and free concentrations of methylene blue were plotted against time to determine the equilibration time

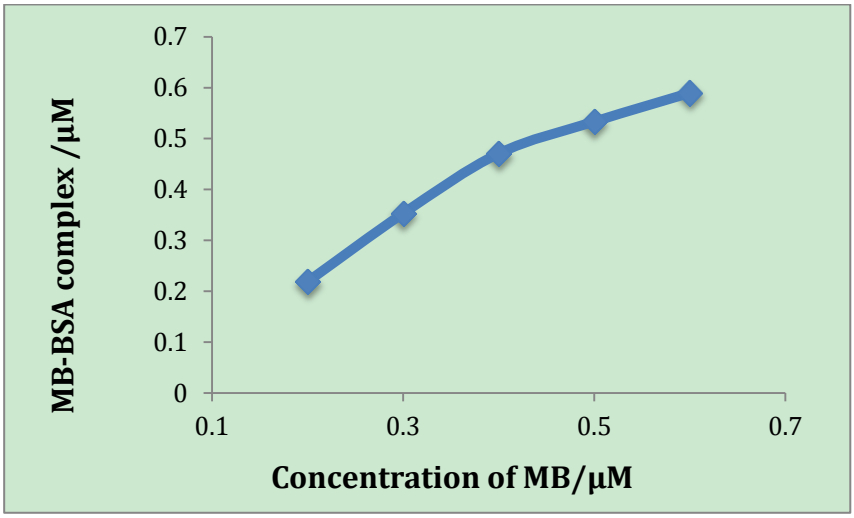


Fig. 6 MB-BSA complex/ µM against concentration (methylene blue) / µM

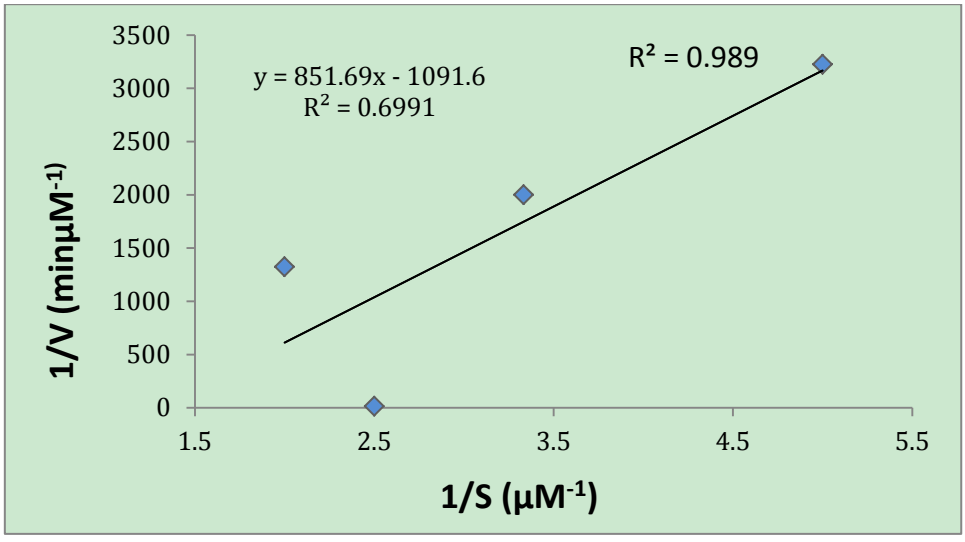


Fig. 7 Double reciprocal plot for the MB-BSA binding. Data shows the relationship between the rate of binding and the MB concentration. Estimates of the Bmax and Kd were obtained from the x-intercept and the slope, respectively

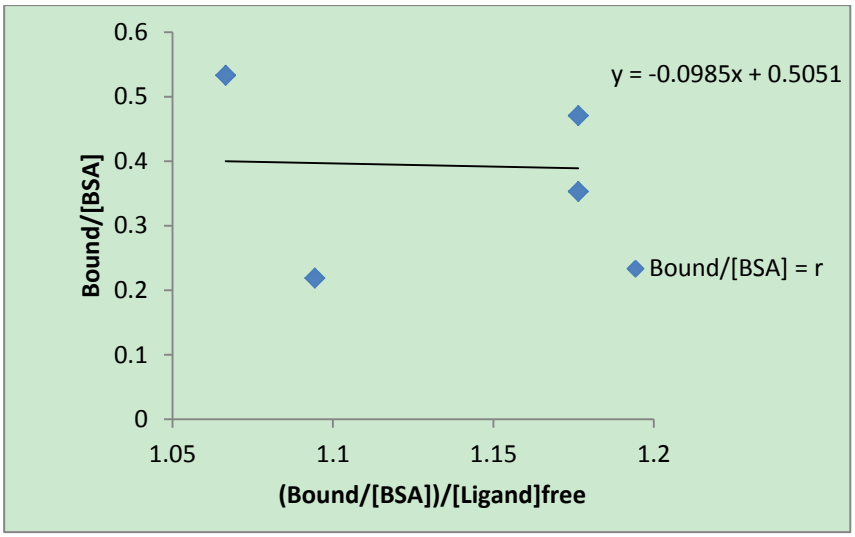


Fig. 8 Scatchard plot for the ligand binding data from which estimates of n and –Kd were obtained from the values of the y intercept and the slope, respectively

F. Evaluation of the Effects of pH on the Binding

For the effects of pH on the MB-BSA binding, about 20 mL of 0.5 µM methylene blue solution at various adjusted pH values, from 5 to 9, was dialyzed against 1 mL of 0.17 µM BSA for 2 hrs. A plot of MB-BSA complex versus pH is represented in Fig. 9.

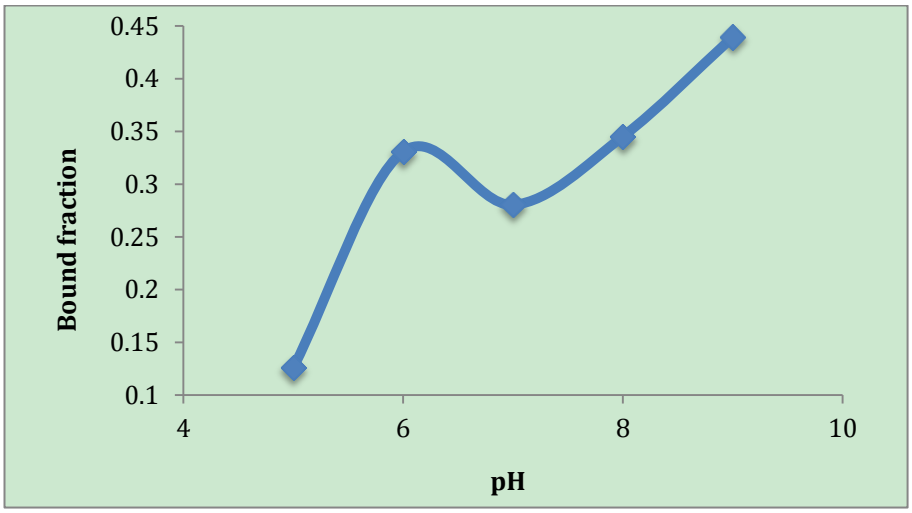


Fig. 9 Bound fraction of methylene blue against pH; By dialyzing 0.5 µM methylene blue against 0.17 µM BSA at varying pH levels, the relationship between the bound fraction of methylene blue and pH was observed, as indicated in the plot

G. Effects of Temperature on the Binding

The effects of dialysis temperature on the binding of methylene blue were studied in the range 20 °C – 45 °C. The temperature of the reaction system was varied from 20 °C – 45 °C for 2 hrs. A graph of MB-BSA complex against different temperatures was plotted and is shown in Fig. 10. The effects of temperature on the rate and equilibrium constants were shown in Figs. 11-14 from which the thermodynamic parameters were evaluated.

H. Effects of Salt Concentrations on the Binding

The effects of salt concentrations on the binding of methylene blue to the BSA was studied by dialysing 0.5 µM methylene blue solution containing varying concentrations of NaCl (200 mM to 1,000 mM) against 0.17 µM BSA containing the same concentrations of salt for 2 hrs. A graph of the bound fraction of methylene blue against salt concentration/mM was plotted, as shown in Fig. 15.

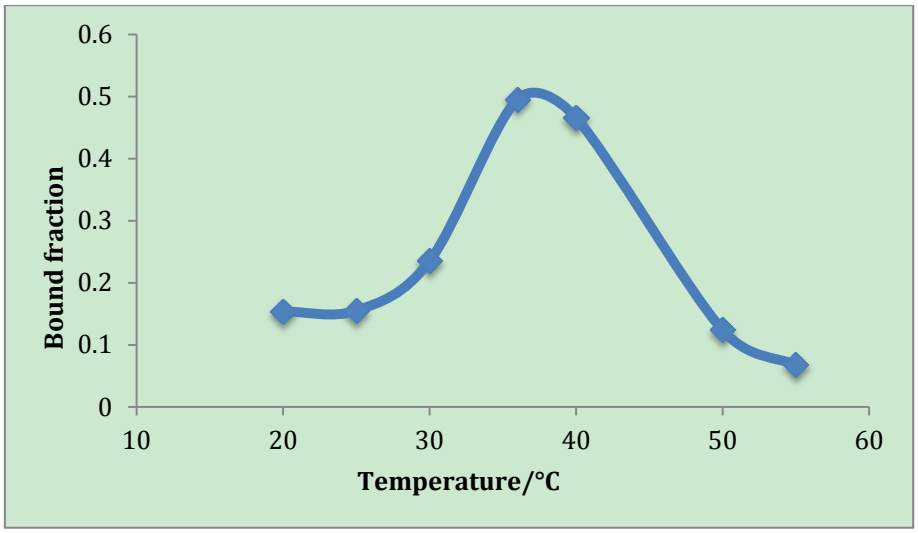


Fig. 10 Bound fraction of methylene blue against temperature. By dialyzing 0.5 µM methylene blue against 0.17 µM BSA at varying temperatures, a relationship between the bound fraction and temperature was observed

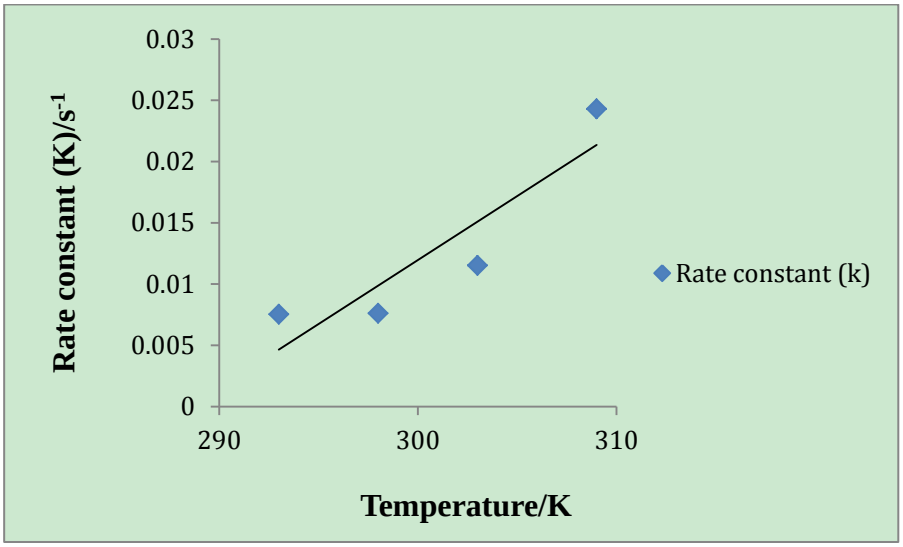
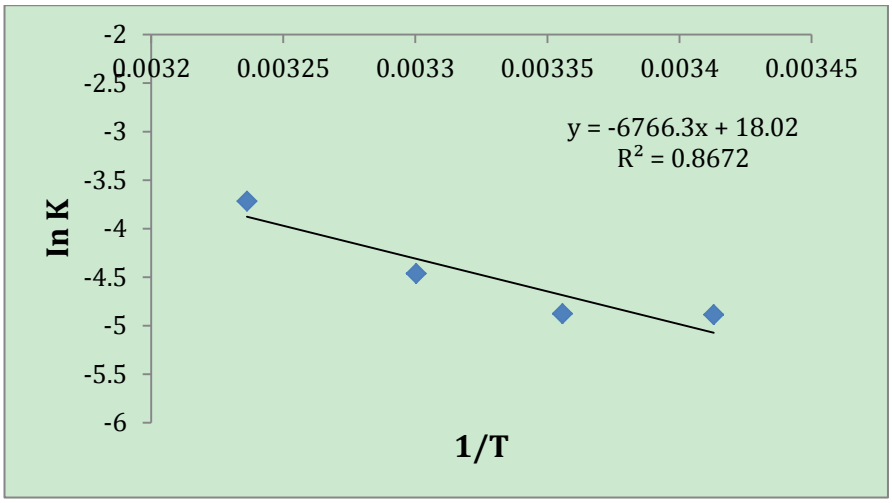


Fig. 11 Change in rate constant with increasing temperature



k=A*exp(-Ea/R*T)

Fig. 12 Arrhenius plot of In K against 1/T

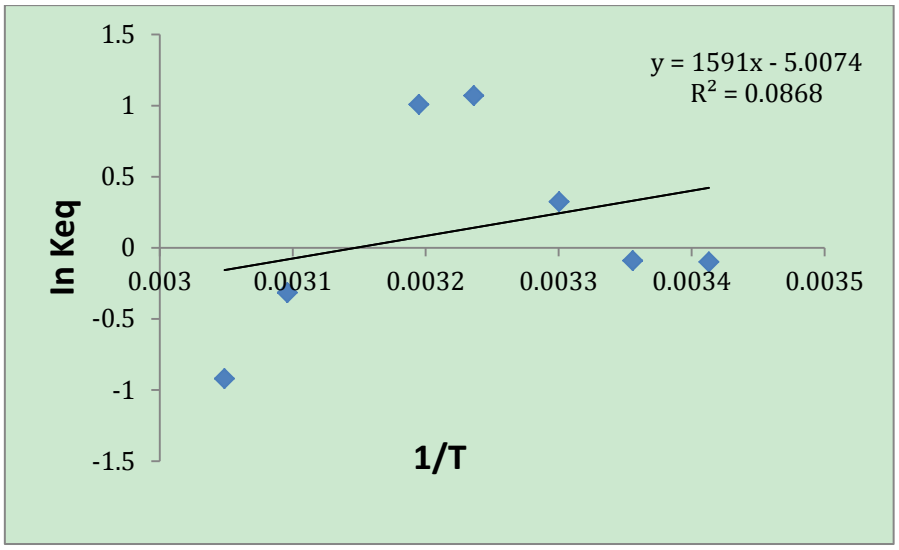


Fig. 13 Van't Hoff plot of In Keq against 1/T; dialysis was performed at varying temperatures, and data in the plot were obtained from the thermodynamic parameters determined

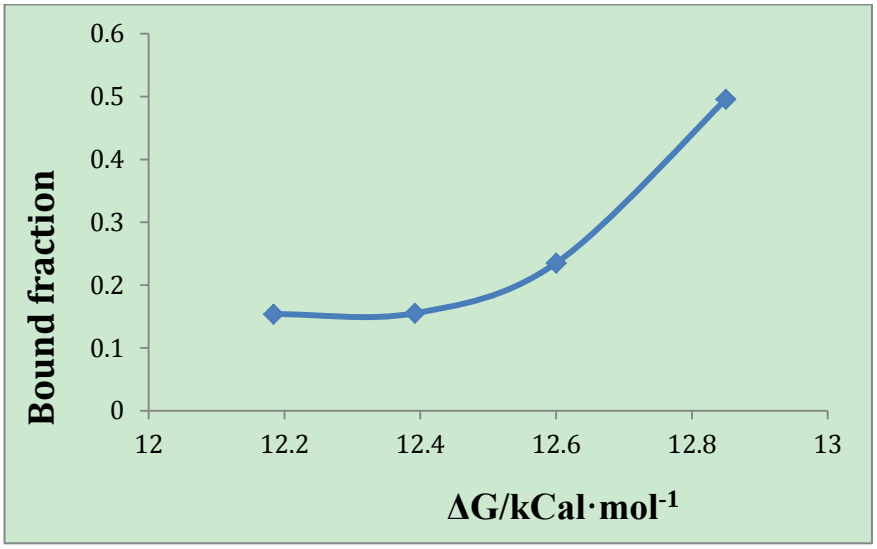


Fig. 14 Effects of increasing ΔG values with increasing temperatures on bound fraction. The ΔG at different temperatures was calculated and plotted against the bound fraction

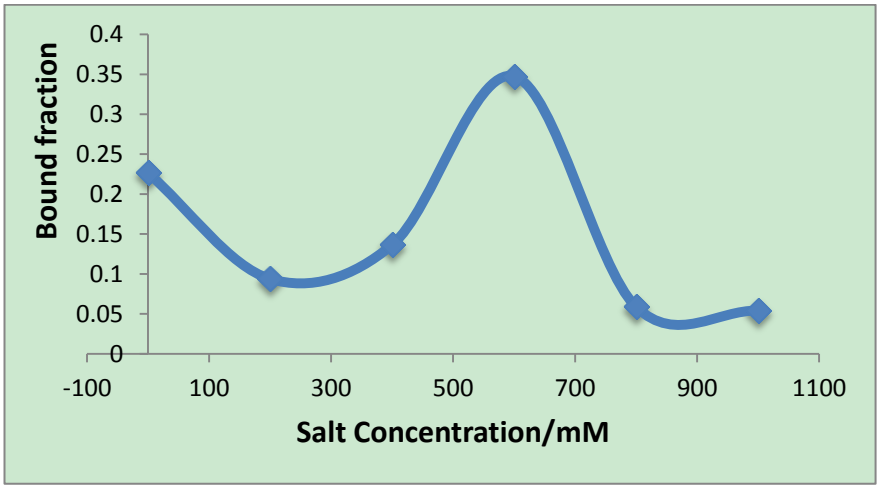


Fig. 15 Bound fraction of methylene blue against salt concentration/mM. 0.5 µM methylene blue solution was dialyzed against 0.17 µM BSA at varying salt concentrations to establish the effect of salts on the binding

I. Data Analysis

The results obtained in the tests were analyzed using Microsoft Excel and Pearson's Correlation. Repeating experiments under the same conditions ensured the reproducibility of the measurements.

III. RESULTS AND DISCUSSION

A. Suitability of the Setup and Establishment of Suitable Working Conditions

In comparison to other equilibrium dialysis apparatus such as the micro-equilibrium dialyzer, multi-equilibrium dialyzer and other protein-ligand binding assays, the setup developed in this study has potential advantages such as requiring less protein, being simpler and cheaper to assemble, and having higher speed. Due to the fact that the quantitation of the binding of MB and BSA was based on measurements of absorption of the MB and attainment of equilibrium, preliminary studies were conducted to establish suitable conditions, including wavelength of maximum absorption, stability of the dye, time for equilibration, and working concentration of the interacting species, in order to optimize the assay. The wavelength of maximum absorption for methylene blue was determined as 664 nm, as can be seen in Fig. 3. This wavelength (664 nm), which was used for all the subsequent spectrophotometric measurements, is comparable to the values reported by [12] and [13], which were 667 nm and 664 nm, respectively.

To ascertain the stability of the methylene blue dye during the dialysis period, the absorbance of each methylene blue solution was taken initially and then again after 3 hrs. The results displayed in Fig. 4 indicate that the MB dye was stable throughout the 3-hour period and thus suitable for the assay. If the dye is unstable, it can deteriorate and give false negative or positive results.

The time required to reach equilibrium between the two dialysis chambers was also investigated because the success of the method depends solely on attainment of equilibrium. As shown in Fig. 5, the bound fraction of the MB increased with time, with a simultaneous decrease in the free ligand until 1.5 hrs at which time the graph leveled off and there was no increase in the bound fraction. As such, an equilibration time of 2 hrs was used for all subsequent readings. The rate of equilibration was closely correlated with the extent of the MB-BSA binding in accordance with Fick's first diffusion law, which states that the rate at which equilibrium is reached is governed by, among other parameters, the difference in concentration at both sides of the membrane [14]. One drawback of the equilibrium dialysis method is the relatively long hours (>20 hrs) often needed to reach equilibrium, which can give rise to degradation of the compounds. Using this modified equilibrium dialysis setup, the time required for the MB to equilibrate between the two chambers of the setup was 1.5 hrs, which was within the period of its stability (3 hrs). The increase in the bound fraction with time within the 1.5 hours is a result of movement of the methylene blue through the membrane into the protein chamber to achieve equilibrium and the binding of the methylene blue (ligand) to the BSA (protein). Therefore, a dialysis time of 2 hrs was used for all subsequent equilibrium dialysis experiments. This short equilibration time compared to equilibration times of 24 and 40 hours reported in studies by [6] and [14], respectively, was ensured in part by constant stirring throughout the assay period and the ease of passage of MB through the membrane. Also, the concentration difference, a higher MB concentration of 0.5 µM against 0.17 µM of BSA provides a gradient that drives the movement of molecules in an attempt to neutralize the concentration difference. In the binding of BSA and nicotinamide adenine dinucleotide (NAD 299), different initial amounts of NAD 299 and BSA were added to both sides of the dialysis membrane to shorten the equilibration time [14]. One study reported the time required for equilibration for methohexital – albumin binding using Dianorm Science Tech apparatus with 20 cells was 1 hour [5]. It can therefore be deduced that different compounds have different affinities towards their target proteins, which explains the different equilibration times that have been reported. Pearson's correlation between the bound ligand (methylene blue-BSA complex) and time was 0.926, which meant that there was a strong positive correlation between the bound ligand and time.

B. Effects of Methylene Blue Concentration on the Binding

Altering the conditions such as pH, temperature, concentration, and ionic strength of a system affects the binding properties of the interacting species. The effects of varying methylene blue concentrations on the binding of methylene blue to BSA are shown in Fig. 6. The concentrations were set below and above 0.5 µM, with increasing bound fraction as the concentration of MB increased. It was obvious that increasing the MB concentration resulted in a corresponding increase in the bound MB concentration, which was in line with the law of mass action. Another possible explanation could be that the lower MB concentrations were not sufficient to bring about major alterations in the structure BSA, accounting for the low bound fractions. Increasing the concentration of the MB might have induced conformational changes in the BSA structure, increasing its affinity for the methylene blue and bringing about high bound fractions. A similar observation was reported in cisplastin-HAS complexes, which increased with increasing cisplastin concentrations [15].

Fig. 7 represents a double reciprocal plot for the binding data from which estimates of B_{max} and K_d were obtained from the values of the x intercept and the slope, respectively. Fig. 8 shows a scatchard plot for the ligand binding data from which estimates of n and $–K_d$ were obtained from the values of the y intercept and the slope, respectively. From Figs. 7 and 8, the K_d, K_a, B_{max} and n were found to be 9.2 µM, 1.087x105M-1, 0.0143 µM/min, and 0.5051 respectively. The K_a value indicated high MB affinity for the BSA. The K_d for rhodamine B-BSA complex was 2.0x10-5M [3], a 50 kDa protein/phenol red was 8.6x10-

5M [16], and dinitrophenol/transthyretin was 6.4x10-8M [7]. This illustrated that K_d values differ from complex to complex. It can thus be inferred that the methylene blue binding to BSA had a low K_d and a corresponding high K_a. The number of binding sites (n) present on the protein was found to be 0.5051 and the Bmax was 0.0143 µM/min. The complex formation was found to be at a ratio of 1:1. In the binding of the methohexital to the albumin, the number of binding sites on the albumin was 1 [5] and in the binding interaction of neutral red and bovine serum albumin, it was n=0.9462 [9].

C. Effects of pH on the Binding

The effects of pH values on the binding were investigated by dialysing 0.5 µM methylene blue against 0.17 µM BSA at varying pH values (from 5 to 9) for 2 hrs, as shown in Fig. 9. Increasing the pH from 5 to 6 resulted in increases in the bound fraction, however, pH levels between 6 and 7 showed the binding decreased. As the pH increased from 7 to 9, further increases in the bound fraction were recorded.

An increase in the bound fraction was observed with increasing pH values, which could be due to the aggregation of the BSA and conformational changes exposing certain sites to which the methylene blue readily binds. This resulted in an increase in affinity of the MB dye for BSA near its isoelectric point (pI), which was around 4.9 or 5 [17]. At pH 7, the bound fraction decreased from 0.0028 to 0.0023. This could be because at a pH value above the pI of the BSA, there was titration of the amino acid side chains leaving the BSA with a negative charge that resulted in a decrease in the bound fraction. Interestingly, as the pH was increased from 7 to 9, the bound fraction increased once again. This may be due to the fact that, at pH levels far higher than the pI of the BSA, there was deprotonation, meaning additional groups might have been titrated to their base forms, which made the BSA molecule more basic. Moreover, the increase in the bound fraction of methylene blue with increases in pH could also be explained by a higher affinity of MB towards the basic (B-form) conformation of the BSA. This was consistent with an increase in the bound fraction of methohexital-albumin when the pH was increased from 7 to 9 [5].

D. Effects of Temperature on the Binding of Methylene Blue to BSA

By dialysing 0.5 µM MB against 0.17 µM BSA at varying temperatures from 20 °C to 56 °C for 2 hours, the effects of different temperatures on the interaction between MB and BSA were investigated, as shown in Fig. 10. As the temperature increased from 20 °C to 36 °C, there was a corresponding increase in the bound fraction, with the highest value being 36 °C. As the temperature increased to 40 °C, there were significant decreases in the bound fraction, until the temperature reached 55 °C.

The effects of temperature on the rate constant are also shown in Fig. 11. Increases in temperature resulted in increases in the rate constant. As the temperature increased from 20 °C to 25 °C and 30 °C, the bound methylene blue concentration increased from 0.0769 µM to 0.0777 µM and 0.233 µM, respectively. Beyond 40 °C, there was a decrease in the bound fraction until the temperature reached 55 °C, at which almost no binding occurred. Increasing the temperature increases molecular motion (faster diffusion), causing collisions between the BSA and methylene blue to increase. This results in a higher kinetic energy, which has an effect on the activation energy of the reaction. The results displayed in Fig. 11 also show an increase in the rate constant with increasing temperatures. At lower temperatures, most molecules do not have enough energy to form complexes. Increasing the temperature increases the number of the very energetic particles, with energies equal to or greater than the activation energy. This is evident in the increase in the bound fraction as temperatures increase. Proteins have optimum temperatures beyond which denaturation occurs and binding of the protein to ligands becomes ineffective, which results in decreases in the bound fraction. A decreased bound fraction can also be due to the disruption of the binding forces; electrostatic interactions, vander Waals interactions (weak intermolecular attraction forces between MB and neighbouring BSA molecules), and hydrogen bonds between the BSA and MB at higher temperatures leads to the dissociation of weakly-bound complexes. In the binding of methohexital to albumin, a decrease in the bound fraction with increasing temperatures (20 – 45 °C) has been reported, as though binding forces diminish with increases in temperature [5]. On the contrary, increased temperatures have reportedly no effect on the bound fraction of thiopental-albumin binding [18]. This could be due to strong hydrophobic interactions between the thiopental and albumin that are not disrupted by increased temperatures.

The activation energy of the binding reaction shown in Fig. 12 and the Arrhenius equation was 56.23 $kCalmol^{-1}K^{-1}$. The Arrhenius plot shown in Fig. 12 shows the effects of activation energy and temperature on the rate constant. The results were used to evaluate the activation energy (E_a) using the following equation:

$$InK = In\ A - \frac{Ea}{RT}$$

where the slope of the line gives E_a and the intercept In A.

The fraction of the reaction molecules with energies equal to or in excess of the activation energy increases with increasing temperatures resulting in an increase in the bound fraction. From a study carried out by [18], the binding constant of oxaprozin–BSA at 298 K was $9.7904x10^{-4}$ $Lmol^{-1}$, which decreased to $6.6x10^{-4}$ $Lmol^{-1}$ and $5.3029x10^{-4}$ $Lmol^{-1}$ when the temperature was increased to 302 K and 310 K, respectively. In this study, Pearson's correlation between the bound MB and

the temperature was 0.903 with a P-value of 0.05, indicating a strong positive correlation between temperature and the bound fraction.

The thermodynamic parameters ΔG, ΔH and ΔS shown in Fig. 13 were 12.392 kCal per mole, -13.227 kCal per mole, and -41.63 $Calmol^{-1}K^{-1}$, respectively. This was a typical exothermic reaction, which was evident of the negative ΔH, indicating that enthalpy was favourable for the binding. While the negative ΔS suggested that the reaction was not entropy-driven, the positive ΔG value indicated that the reaction was not spontaneous. The thermodynamic parameters, such as ΔG, ΔH, and ΔS were estimated from the equation In Keq= (-ΔH/RT) + ΔS/R, as shown by the Vant Hoff's plot in Fig. 13 where the slope of the line presents ΔH and the intercept presents ΔS. ΔG was obtained using the equation ΔG = ΔH - TΔS. The effects of increasing ΔG values on the binding were evaluated. It was observed that increasing temperatures resulted in increasing ΔG and also an increase in the bound fraction, as shown in Fig. 14.

E. Effects of Salt on the Binding of Methylene Blue to BSA

To minimize the Donnan effect on the binding, NaCl of varying concentrations was added to the buffer used for the dialysis. The effects of the salt on the binding are illustrated in Fig. 15. From Fig. 15, it can be seen that at a salt concentration of 200 mM, the bound fraction decreased from 0.227 (salt concentration 0 mM) to 0.094. This may be a result of shielding the charges on the methylene blue and BSA by the ions present in the salt, which reduced the Donnan effect. The initial increase in the bound fraction of the methylene blue in the blank may not be due to the binding but as a result of the ion gradient between the two chambers (migration into the protein chamber of the methylene blue dye to attain neutrality). As the salt concentration increased from 400 mM through to 600 mM, the bound fraction increased to 0.136 and 0.347, respectively. This can be attributed to the "salting out" effect of NaCl on the hydrophobic side chains, which can induce structural changes in the binding sites. Such changes may increase the hydrophobic interactions and the binding affinity for the ligand. Increasing the salt concentration from 600 to 800mM demonstrated a drastic decrease in the bound fraction. Salt concentrations in the range of 800 mM to 1,000 mM did not change the level of the bound fraction. This may be due to the fact that the extremely high salt concentrations resulted in increased ions that interacted directly with the polar groups (amide groups) in the proteins, resulting in the formation of more soluble complexes (based on ion-dipole interactions) and thus decreasing the affinity of the protein for the methylene blue. Thus, it can be inferred that there is a minimum concentration of salt that may cause an increase in the affinity of the protein to the ligand, as well as a maximum concentration of salt, which reduces affinity and makes binding ineffective. While determining the effects of salt on protein DNA interaction, the observed increases in equilibrium binding constant with increasing salt concentrations were a result of the removal of large numbers of water molecules from the buried hydrophobic surface area of the protein, thus increasing its affinity for the DNA molecule [19].

IV. CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated the setup's ability to aid in studying binding of small molecules to macromolecules. The K_d, K_a, B_{max}, and n were found to be 9.2 μM, $1.087x10^5M^{-1}$, 0.0143 μM/min, and 0.5051 respectively. Additionally, the thermodynamic parameters ΔG, ΔH, and ΔS were found to be 12.392kCal, -13.227 kCal, and -41.63 $Calmol^{-1}K^{-1}$, respectively. In conclusion, the most interesting point worth noting is how this simple method makes it possible to measure kinetic and thermodynamic parameters, resulting in graphs that conform to characteristics of protein-ligand interactions. Furthermore, these results suggest that the setup can be used to conveniently detect and characterize protein-ligand interactions in any basic laboratory.

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List of abbreviations

ED	Equilibrium dialysis
MB	Methylene Blue
BSA	Bovine Serum Albumin
ΔG	Gibbs free energy change
ΔH/ΔS	Enthalpy / Entropy change

REFERENCES

[1] P. Liu, J. Zhang, C. N. Ferguson, H. Chen, and J. A. Loo, "Measuring protein-ligand interactions using liquid sample desorption

electrospray ionization mass spectrometry," *Analytical Chemistry*, vol. 85, pp. 11966-11972, 2013.

[2] U. Kragh-Hansen, E. Dørge, and A. O. Pedersen, "Rate-of-dialysis technique: Theoretical and practical aspects," *Analytical Biochemistry*, vol. 340, pp. 145-153, 2005.

[3] H. H. Cai, X. Zhong, P. Yang, W. Wei, J. Chen, and J. Cai, "Probing site-selective binding of rhodamine B to bovine serum albumin," *Colloids and Surfaces A: Physicochem. Eng. Aspects*, vol. 372, pp. 35-40, 2010.

[4] A. Raz, T. Shiratori, and D. S. Goodman, "Studies on the Protein-protein and protein-ligand interactions involved in retinol transport in plasma," *The Journal of Biological Chemistry*, vol. 245, pp. 1903-1912, 1970.

[5] I. Girard and S. Ferry, "Protein binding of methohexital: Study of parameters and modulating factors using the equilibrium dialysis technique," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 14, pp. 583-591, 1996.

[6] H. Sillén, M. Cook, and P. Davis, "Determination of unbound ticagrelor and its active metabolite (AR-C124910XX) in human plasma by equilibrium dialysis and LC–MS/MS," *Journal of Chromatography B*, vol. 879, pp. 2315-2316, 2011.

[7] Y. Xie, D. Zhang, and D. Ben-Amotz, "Protein–ligand binding detected using ultrafiltration Raman difference spectroscopy," *Analytical Biochemistry*, vol. 373, pp. 154-160, 2008.

[8] I. Kariv, H. Cao, and K. Oldengurg, "Development of a High Throughput Equilibrium Dialysis Method," *Journal of Pharmaceutical Sciences*, vol. 90, pp. 580-587, 2001.

[9] X. Liu, X. Wu, and J. Yang, "Protein determination using methylene blue in a synchronous fluorescence technique," *Talanta*, vol. 81, pp. 760-765, 2010.

[10] Y. Hu, Y. Liu, R. Zhao, J. Dong, and S. Qu, "Spectroscopic studies on the interaction between methylene blue and bovine serum albumin," *Journal of Photochemistry and Photobiology A: Chemistry*, vol. 179, pp. 324-329, 2006.

[11] Y. Li, Y. Zhang, S. Sun, A. Zhang, and Y. Liu, "Binding investigation on the interaction between Methylene Blue (MB)/TiO_2 nanocomposites and bovine serum albumin by resonance light-scattering (RLS) technique and fluorescence spectroscopy," *Journal of Photochemistry and Photobiology B: Biology*, vol. 128, pp. 12-19, 2013.

[12] B. Liu, Y. Guo, J. Wang, R. Xu, X. Wang, D. Wang, L. Zhang, and Y. Xu, "Spectroscopic studies on the interaction and sonodynamic damage of neutral red (NR) to bovine serum albumin (BSA)," *Journal of Luminescence*, vol.130, pp. 1036-1043, 2010.

[13] J.P. Tardivo, A. D. Giglio, C. S. Oliveira, D. S. Gabrielli, H. C. Junqueira, D. B. Tada, D. Severino, R. F. Turchiello, and M. S. Baptista, "Methylene blue in photodynamic therapy: from basic mechanisms to clinical applications," *Photodiagnosis and Photodynamic Therapy*, vol. 2, pp. 175-191, 2005.

[14] M. A. L. Eriksson, J. Gabrielsson, and L. B. Nilsson, "Studies of drug binding to plasma proteins using a variant of equilibrium dialysis," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 38, pp. 381-389, 2005.

[15] J. F. Neault and H. A. Tajmir-Riahi, "Interaction of cisplatin with human serum albumin: drug binding mode and protein secondary structure," *Biochimica et Biophysica Acta*, vol. 1384, pp. 153-159, 1998.

[16] R. O'Brien, B. DeDecker, G. K. Fleming, P. B. Sigler, and J. E. Ladbury, "The effects of salt on the TATA binding protein-DNA interaction from a hyper-thermophilic archaeon," *Journal of Molecular Biology*, vol. 279, pp. 117-125, 1998.

[17] D. Voet and D. G. Voet, *Biochemistry*, 2nd ed., John Wiley & Sons, Inc., pp. 48-49, 1995.

[18] A. R. Bjorksten, D. P. Crankshaw, D. J. Morgan, and P. R. Prideaux, "Studies of drug-protein binding," *Journal of Cardiothoracic Anesthesia*, vol. 2, pp. 281-282, 1988.

[19] S. Shaofa and X. Guangya, "Molecular spectroscopic studies on the interaction between oxaprozin-P and bovine serum albumin," *Chemistry mag*, vol. 9, pp. 6-25, 2007.