Thamar University Journal of Natural & Applied Sciences 9(1) (2024) 1 – 6

DOI: https://doi.org/10.59167/tujnas.v9i1.2047

TUJNAS

Original Article

Competitive Binding of Methylene Blue to Calf Thymus DNA and Carrageenan: Implications for Sensor Development

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Received: 30 January 2024. Received (in revised form): 10 March 2024. Accepted 16 March 2024. Published 26 June 2024.

Abstract

Carrageenans are sulfated polysaccharides widely used in the food industry due to their excellent thickening, stabilising, and gelling properties. **Objective:** This study delves into two key aspects: 1) the binding interaction between the cationic dye methylene blue (MB) and carrageenan compared to other polyanions (alginate, starch, and Arabic gum), and 2) the competitive binding behavior of carrageenan and DNA for MB. **Methods:** Spectroscopy methods were employed to determine the total amount of carrageenan by utilising MB, which creates complexes with carrageenan. The investigation into the competitive binding between polysaccharides and double-stranded DNA modified with screen-printed electrodes (SPEs) (dsDNA/SPE) for methylene blue (MB) involved the addition of varying quantities of polysaccharide to a solution containing 100 µM MB after a 5-minute accumulation period. Subsequently, cyclic voltammograms were directly recorded to analyse the interactions. **Results:** Strong electrostatic binding between MB and carrageenan was confirmed using various techniques, including UV-vis, fluorescence, and cyclic voltammetry. Moreover, photoluminescence (PL) spectroscopy was more sensitive than Ultraviolet-visible (UV-vis) spectroscopy, detecting carrageenans in a linear range from 0.5-15 ppm, 2-15 ppm, with correlation coefficients 0.998, 0.997, respectively. Notably, the preference for binding increased with increasing sulfate content, following the order kappa > iota > lambda carrageenan, highlighting the crucial role of sulfate groups in complex formation. Additionally, carrageenan exhibited the strongest competitive binding towards MB at low concentrations compared to other tested polyanions, implying its effectiveness in hindering MB-DNA interactions. **Conclusions:** These findings pave the way for exciting developments in DNA biosensors. The sensitive colorimetric assay holds promise for the rapid and selective determination of carrageenan levels in food products. Moreover, the competitive binding between carrageenan and MB can be harnessed in DNA biosensors by monitoring changes in MB binding due to specific DNA sequences.

Keywords: Carrageenans; Competitive Binding; Calf Thymus; Double-Stranded DNA; Spectroscopy; Cyclic Voltammetry

1. Introduction

Food scientists appreciate polysaccharide hydrogels for their exceptional thickening and gelling properties, making them preferred ingredients for modifying the texture and characteristics of delicious treats. The significance of polysaccharide hydrogels extends to various industries, with a particular emphasis on their applications in food and medicine [1]. These hydrogels are commonly employed to gel and thicken foods, exerting influence over the flow and functionality of food systems [2]. Unlike proteins and nucleic acids, polysaccharides have repeated structures of sugar units joined together by glycosidic linkages. Their capacity for structural variability positions polysaccharides as highly adept carriers of biological information, rendering them particularly intriguing in biochemistry and medicine [3, 4]. Certain polysaccharides derived from seaweeds manifest diverse biological effects [5]. Furthermore, sulfated polysaccharides showcase properties such as anticoagulant, antiviral, and immuno-inflammatory activities, suggesting potential applications in health foods, cosmetics, and pharmaceuticals [6, 7].

The negative charge imparted by the sugar-phosphate backbone renders calf thymus DNA polyanionic, establishing nucleic acids as capable tools for the detection and quantification of various significant substances [8]. The interaction between transition metal complexes and the nitrogenous bases of DNA is a subject of considerable interest [9-11]. These complexes can engage with DNA through covalent binding, electrostatic interactions, or intercalation [12]. The specific ligand and metal employed play a crucial role in determining how these complexes interact with the DNA molecule, offering insights for developing novel drugs and designing more efficient DNA recognition and cleavage agents [13, 14]. Methylene blue (MB), an organic dye belonging to the phenothiazine family, exhibits reversible electron transfer reactions [15]. It also functions as an aromatic heterocyclic compound with strong intercalation capabilities with DNA. This intercalation occurs between the double-stranded DNA (dsDNA) base pairs and MB. The positive charge of MB enhances its affinity for DNA through electrostatic interactions with the phosphate backbone [16].

The intricate interactions between biopolymers and small molecules like dyes are essential in various fields, ranging from biosensor development to fundamental biological research [17]. This study investigates the competitive binding interactions between ds-DNA and various polysaccharides using methylene blue (MB) as a probe, particularly emphasizing the influence of carrageenan's sulfation level. The research employs a quantitative approach to assess the impact of polysaccharides on ds-DNA activity through pre-exposure of ds-DNA to varying carrageenan concentrations before MB accumulation. While traditional MB-polyanion interactions offer limited quantitative information, this work focuses on the interferential effects between MB-carrageenan and other polysaccharide interactions and the competition between MB-DNA and carrageenan. Three analytical techniques are employed: UV-Vis spectroscopy, photoluminescence (PL) spectroscopy, and cyclic voltammetry (CV). This comprehensive approach allows for a detailed investigation of MB-DNA complexation and its modulation by various polysaccharides. Furthermore, the study aims to translate the concept of competitive binding into developing biosensors for carrageenan detection. By leveraging the amperometric method, this work proposes a novel approach for quantifying carrageenan based on its interference with MB-DNA interactions.

2. Materials and Methods

2.1 Chemicals and Apparatus

Acros Organics and Duchefa Biochemie supplied Tris(hydroxymethyl) aminomethane (Tris-HCl), Riedel-de Haen supplied hydrochloric acid 37% (HCl), Sigma supplied sodium chloride (NaCl) and double-stranded calf thymus DNA (dsDNA, activated and lyophilized, number 4522). 5 mg of calf thymus dsDNA was dissolved in 1 ml of TE buffer containing 10 mM Tris-HCl (pH 8) and 1 mM EDTA, then frozen. 10 mM Tris-HCl Buffer and 10 mM NaCl at pH 7 were used to prepare dilute dsDNA solutions. R & M Chemicals supplied methylene blue (MB). A 1 mM MB stock solution was prepared in water and stored at 4℃ in the dark. More solutions were prepared by diluting 10 mM Tris-HCl and 10 mM NaCl to pH 7. Sigma supplied the carrageenan types, ι-(iota) and λ-(lambda), and Fluka supplied the κ-(kappa) type (which contains calcium alginate, starch, and gum arabic). 50 mg of carrageenan was dissolved in 50 mL MilliQ water (18 MOhm) in a bath at 50℃. The solutions were prepared by diluting 10 mM Tris-HCl and 10 mM NaCl to room temperature with pH 7. The other compounds were of analytical reagent grade. MilliQ water with 18 MOhm resistance was used to prepare the solutions.

To obtain the UV-Vis absorption spectra, a Cary 100 model Varian spectrophotometer and a 10 mm quartz cell were used. The CV measurements were performed using a Metrohm Autolab PGSTAT302N instrument with GPES 4.9 software by Eco-Chemie in the Netherlands. The working electrode was a screen-printed (SPE) from Scrint Technology (M) Sdn. Bhd. The reference electrode was Ag/AgCl (3M KCl), and the counter electrode was glassy carbon. These formed the three-electrode system used in this study.

2.2 Spectrophotometric and Electrochemical Studies

The UV-Vis absorption spectra were obtained by adding different amounts of polyanion to 10 µM MB. The competitive binding of polyanion and ds-DNA to MB was studied by adding different amounts of polyanion to the dsDNA-MB system. The MB and DNA concentrations were fixed, and the changes in the spectrum intensity of dsDNA-MB were measured. The experiment involved applying 3 µL of the stock solution to immobilize calf thymus dsDNA on the SPE surface. No electrochemical preconditioning was done, and the electrode was dried overnight.

To remove any unbound dsDNA on the working electrode and ensure a uniform DNA coating, the ds-DNA/SPE was immersed in a 10 mM Tris-HC buffer for 5 minutes. The dsDNA-SPE-modified electrodes were then immersed in a 100 µM MB solution. The electrodes were kept on an open circuit without any potential applied for 5 minutes of accumulation time. The competitive binding of polysaccharide and ds-DNA to MB was studied by adding different amounts of polysaccharide to a 100 µM MB solution after 5 minutes of accumulation time and then recording the cyclic voltammograms directly.

3. Results and Discussion

3.1 Polyanion /Methylene Blue Interaction

The UV-Vis absorption spectra of MB at a concentration of 10 µM in aqueous solution revealed a strong dependence on its concentration due to a monomer-dimer equilibrium. The characteristic peaks for MB monomer and dimer are located at 664 nm and 614 nm, respectively, as highlighted in the inset of Figure 1. The clear distinction between these species through absorption spectroscopy has been established in previous studies [18]. Interestingly, Cenens [19] identified four MB species on clays: monomer (MB^+), protonated monomer (MBH^{2+}), dimer ($(MB^+)_2$), and trimer ($(MB^+)_3$), suggesting these might also be present in solution.

At 10 µM, MB predominately exists as a monomer, as evidenced by the dominant peak at 664 nm (Figure 1). However, increasing MB concentration significantly affects its aggregation state. Monomer abundance (λ_{max} = 664 nm) decreases in favour of higher-order aggregates with peaks between 605 and 585 nm, resulting in a gradual red shift of the maximum absorption wavelength from 605 to 585 nm. Significantly, the intensity of the dimer peak ($\lambda_{max} \sim 610$ nm) increases with increasing MB concentration, reflecting its aggregation dynamics. While dimers may be present at low concentrations, their observation is often hindered by the limited sensitivity of UV-Vis spectrophotometry [20, 21].

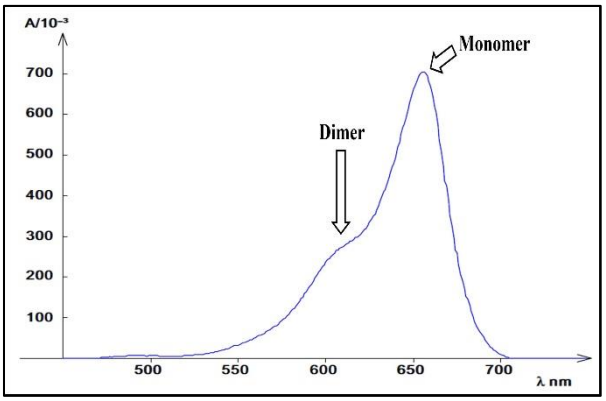

Figure 1: Absorption spectrum of methylene blue. The spectrum shows the absorption of MB in 10 µM Tris-HCl buffer at pH 7. A cell with a path length of 1 cm was used to measure the spectrum.

Alterations in the absorption spectrum characterise the interaction between MB and various polyanions. This change generally arises from the accumulation of dye molecules near the polymer chain, leading to the formation of dye aggregates. However, the observed effects vary depending on the specific polyanion involved. As illustrated in Figure 2a-c, alginate, starch, and Arabic gum display minimal alterations in the MB absorption spectrum, suggesting weak or negligible interaction with the dye. In contrast, carrageenan exhibits a distinct response upon binding with MB. As shown in Figure 2d, a new metachromatic band emerges at 554 nm, shifting from blue towards purple in the overall spectrum. This phenomenon, previously reported by Soedjak [22], signifies the formation of a complex between MB and carrageenan.

Cationic dyes, such as MB, interact with polyanions to form complexes characterised by metachromatic bands, which is evident in the absorption spectrum as it shifts towards shorter wavelengths. As shown in Figure 3, the presence of carrageenan in the solution induces a noticeable shift in the MB absorption spectrum towards a shorter wavelength while simultaneously decreasing the intensity of the monomer peak at 664 nm and the dimer peak at 610 nm. This observation suggests the formation of a metachromatic complex between MB and carrageenan, evidenced by the increasing peak intensity at 554 nm, corresponding to the purple colour observed.

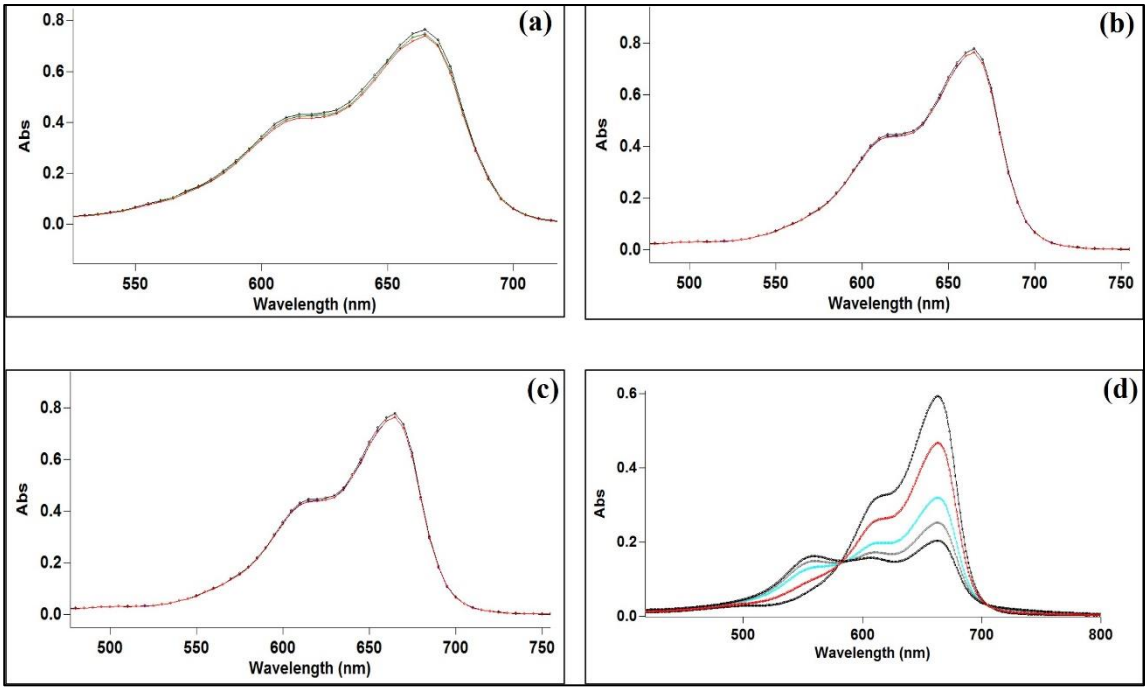

Figure 2: Absorption spectrum of methylene blue with different polyanions (5 ppm, 10 ppm, 15 ppm, and 20 ppm): (a) alginate, (b) starch, (c) Arabic gum, and (d) λ-carrageenan. The solutions were made in 10 mM Tris-HCl buffer at pH 7.0 with 10 mM NaCl.

Figure 3 further reveals a decrease in the maximum peak intensity (λ_{max}) of free MB in the presence of different carrageenan types, notably in the order of κ, ι, and λ-carrageenan. This trend aligns with these carrageenans' increasing sulfate content (SO_3^{-2}), with λ-carrageenan possessing the highest charge density due to its abundant sulfate groups. This observation suggests a correlation between sulfate content and MB binding affinity, reinforcing previous findings by Soedjak [22] that carrageenan-MB interactions increase with increasing sulfate content. It is important to note that the interaction between methylene blue and polysaccharides is primarily electrostatic and reversible, highlighting the role of ionic interactions in complex formation. The absorption peak at 554 nm increases linearly with carrageenan concentrations up to 15 ppm, reflecting the formation of the MB-carrageenan complex. At higher concentrations, the curve plateaus, suggesting the complete consumption of free MB in the reaction.

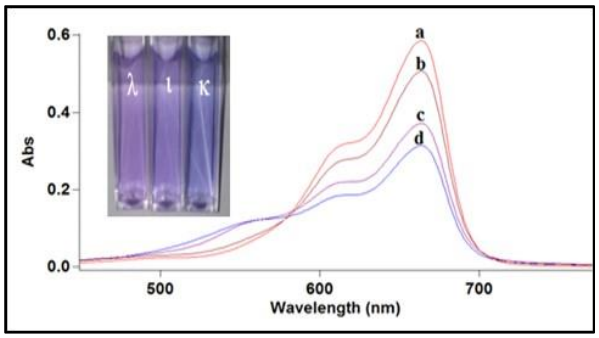

Figure 3: Absorption spectrum of methylene blue with different carrageenans (10 ppm) a) MB, b) κ-carrageenan, c) ι-carrageenan and d) λ-carrageenan. The carrageenans were added separately to 10 μM MB in 10 mM Tris-HCl buffer at pH 7.0.

Interestingly, the decrease in absorbance at 664 nm (corresponding to free MB) shows excellent linearity with increasing carrageenan content (correlation coefficient of 0.997). This high degree of linearity enables the efficient determination of unknown carrageenan concentrations through a simple absorbance measurement at 664 nm. In addition to its absorption properties, MB exhibits fluorescence with an emission maximum of 680 nm. Studies on the MB-polyanion system revealed that the fluorescence intensity of MB decreases with increasing polyanion concentration. Notably, fluorescence spectroscopy proved to be significantly more sensitive for detecting carrageenan-MB interactions, with a linear detection range spanning 0.5-15 ppm compared to 2-15 ppm for UV-Vis. This enhanced sensitivity allows for accurately determining even trace amounts of carrageenan via pre-constructed calibration curves based on fluorescence intensity measurements.

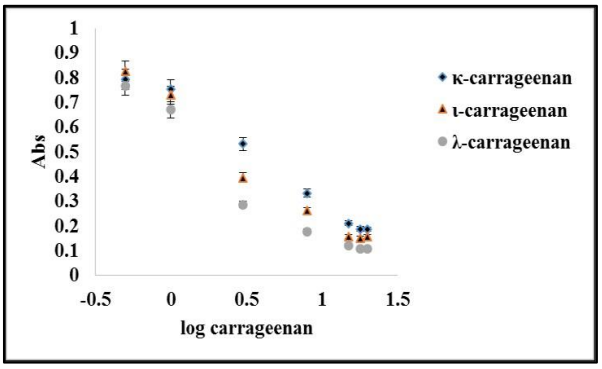

Figure 4: The maximum absorption peak intensity of the free MB absorption spectrum with different concentrations of κ-carrageenan, ι-carrageenan, and λ-carrageenan. The carrageenans were added separately to 10 μM MB in 10 mM Tris-HCl buffer at pH 7.0.

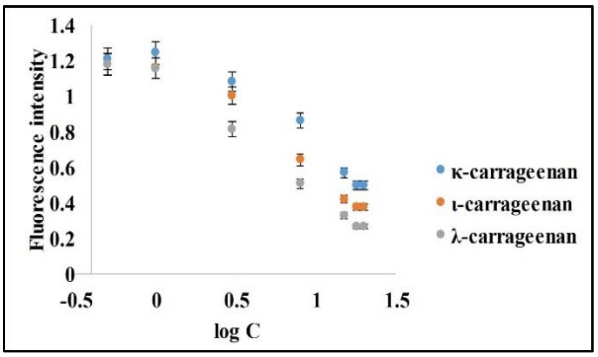

Figure 5: The emission intensity of the free MB emission spectrum with different concentrations of κ-carrageenan, ι-carrageenan, and λ-carrageenan. The carrageenans were added separately to 10 μM MB in 10 mM Tris-HCl buffer at pH 7.0.

There was no interference in the result of the fluorescence method for detecting carrageenan using MB in the presence of other compounds. The emission characteristics of certain dyes can also be used to study the

properties of polymers in general and, in particular, polyanion. The emission at 680 nm decreases linearly as carrageenans reach a concentration of 15 ppm. With increasing polymer content, the curve saturates, possibly due to the total free dye in the reaction— the linearity of the 680 nm decrease in carrageenan content has a correlation coefficient of 0.998. Metachromatic complexes with a purple color are formed quickly by the interaction between MB and carrageenan at low reactant concentrations. The reaction mechanism shown in Figure 6 requires two conditions for forming the metachromatic complex. (1) Polar interaction between the dye and sequential groups on the polyanion (2) Van der Waals interaction between neighbouring dye molecules. The formation and stability of the metachromatic complex depend on how well these interactions can work together and keep the dye molecules in order [23].

The fluorescence method employing MB for carrageenan detection proved remarkably resistant to interference from other compounds, showcasing its specificity towards the target molecule. This characteristic makes it advantageous for real-world applications where complex sample matrices might be encountered. Beyond carrageenan detection, the emission characteristics of MB hold the potential for elucidating the properties of various polymers, particularly polyanions. The decrease in MB emission at 680 nm observed in this study exhibits a linear relationship with carrageenan concentration up to 15 ppm, mirroring the trend seen in UV-Vis absorption. This saturation at higher concentrations likely reflects the reaction's complete consumption of unbound MB. Similar to the UV-Vis results, the decrease in fluorescence at 680 nm displays exceptional linearity with increasing carrageenan content (correlation coefficient of 0.998), offering another avenue for accurately quantifying unknown carrageenan concentrations.

3.2 Rapid Complex Formation and Mechanistic Insights

The interaction between MB and carrageenan is characterised by the rapid formation of metachromatic complexes exhibiting a distinct purple colour at low reactant concentrations. Figure 6 proposes a mechanism for this complexation, highlighting two mutually essential factors: polar interactions. The initial step involves polar interactions between the positively charged MB and the negatively charged sulfate groups on the carrageenan chain. Van der Waals forces are followed by establishing interactions between adjacent MB molecules, promoting self-assembly, and stabilising the complex. The stability and extent of the metachromatic complex depend on the combined influence of these two sets of interactions and their ability to operate in synergy while maintaining the ordered arrangement of MB molecules, as Schoenberg [23] described.

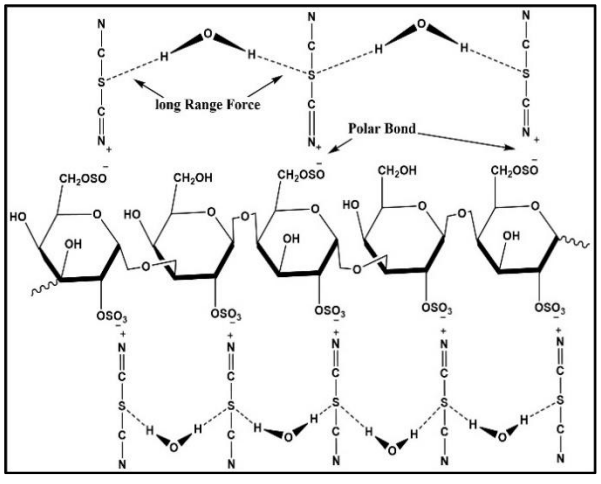

Figure 6: proposed the mechanism of MB and λ-carrageenan in solution.

The specific functional groups on the λ-carrageenan play a crucial role in determining the feasibility of complex formation. As mentioned earlier, the spacing and arrangement of these groups should promote a linear alignment of MB molecules with appropriate interplanar distances to facilitate the interactions. The λ-carrageenan with functional groups naturally spaced within the optimal range or capable of adopting configurations achieving this spacing offers ideal templates for forming stable metachromatic complexes with planar MB.

3.3 DNA Interaction with Methylene Blue

The ability of MB to bind to the number of anionic sites in DNA was initially explored [24, 25]. These studies utilised MB as the probe molecule due to its well-defined interaction with DNA and characteristic spectral changes upon binding. By monitoring the changes in MB's absorption spectrum upon titration with DNA, researchers could estimate the number of available binding sites on the DNA molecule. The interaction between MB and single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) in solution has been extensively investigated using UV-Vis spectroscopy.

Upon addition of increasing amounts of DNA, the peak at 664 nm exhibits a shift towards 668 nm, accompanied by a decrease in absorbance intensity. This shift is attributed to forming an MB-dsDNA complex, with the blue-to-red shift indicating a change in the electronic environment around the dye molecule. The observed shift typically ranges from 3-4 nm (664 nm to 667-668 nm), as reported by Hajian and Tong [26, 27]. Interestingly, the magnitude and nature of the spectral shift observed upon MB binding vary depending on the type of DNA. It suggests that ssDNA and dsDNA interact with MB differently, potentially involving different binding modes. Further investigations are needed to elucidate the specific binding geometries and the underlying mechanisms responsible for these variations in spectral response.

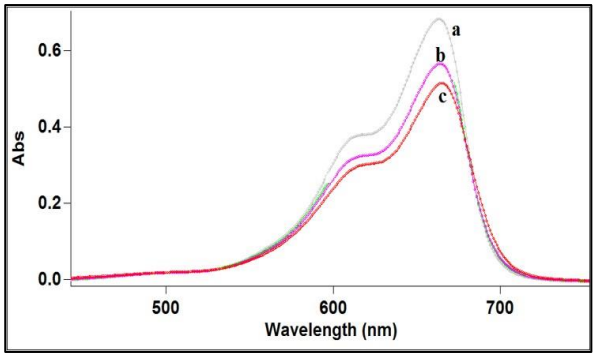

Figure 7: Visible spectrum of 10 µM methylene blue interacts with 10 ppm DNA a) MB, b) ssDNA, c) dsDNA. Add DNA separate to10 µM MB in 10 mM Tris-HCl buffer pH 7.0.

Small molecules can bind to double-helix DNA through three primary mechanisms without forming covalent bonds, as illustrated in Figure 8. First, electrostatic interaction involves electrostatic forces with DNA's negatively charged sugar-phosphate backbone. Second, groove binding entails interactions with the grooves of the DNA structure. Third, intercalation involves the insertion of molecules between the base pairs of the DNA double helix. While grooves in the DNA are implicated in both intercalative and groove binding, electrostatic binding can occur externally to the grooves. Among these modes, intercalative binding stands out as the most effective for drugs targeting DNA, and it is closely associated with the antitumor activity of the compound [28]. Conversely, small molecules labeled MB exhibit three distinct DNA binding modes. These modes include electrostatic interaction with the negatively charged DNA backbone, groove-binding interactions with both the minor and major grooves of DNA and intercalation within the DNA double helix between the base pairs, as depicted in Figure 8 [29-31].

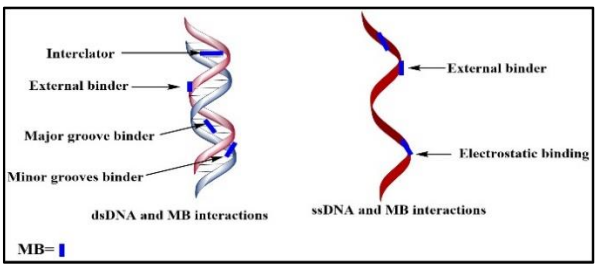

Figure 8: Scheme of different basic interaction mechanisms of MB and DNA (ssDNA, dsDNA).

In conclusion, spectroscopic techniques, particularly UV-Vis spectroscopy, have proven valuable in exploring the binding interactions between MB and DNA. These studies have provided insights into the number of available binding sites, the formation of MB-DNA complexes, and the potential variations in binding modes depending on the DNA type. This knowledge paves the way for further exploration of MB-based sensing and targeting strategies for various biological and synthetic systems.

3.4 The Competitive Interaction of Polyanion with MB-DNA

The study explores the competitive interaction between polysaccharides and preformed MB-dsDNA complexes using cyclic voltammetry (CV) on screen-printed electrodes (SPEs). This approach provides valuable insights into the binding and competitive displacement of MB from dsDNA. The dsDNA is adsorbed onto the carbon surface of the SPE, forming thin films. Overnight drying allows the dsDNA to condense and create complex network structures called DNA lattices, held together by a multitude of non-covalent interactions, including hydrogen bonding, base stacking, electrostatic forces, Van der Waals forces, and hydrophobic interactions [32]. The dsDNA layer on the SPE is then saturated with MB molecules. Unbound MB is removed with a buffer solution, resulting in a stable MB-dsDNA complex on the electrode surface. The CV response reveals a significant increase in peak currents following MB adsorption, reaching a plateau within 5 minutes and indicating successful complex formation.

The main part of the investigation involves adding polysaccharide solutions to the SPE modified with the MB-dsDNA complex (Figure 9: (a) alginate, (b) starch, (c) Arabic gum, and (d) λ-carrageenan). In Figure 9 (d), a notable reduction in the MB signal was observed with an increase in the concentration of λ-carrageenan. The effect was demonstrated by introducing one drop of 1000 ppm carrageenan into a 3 mL solution containing 100 mM methylene blue. The resulting carrageenan concentrations were measured at 3.4, 6.7, 10, and 13.3 ppm. Following the addition of the polyanion, cyclic voltammetry (CV) was conducted after a two-minute interval. The CV diagram for the alternative polyanion exhibits a marginal decrease, suggesting that the oxidised and reduced forms of MB are associated with the DNA, yielding a heightened signal attributable to free MB in the solution and its interaction with the double-stranded DNA layer on the solid-phase electrode (SPE). While the MB electrode was immersed in the carrageenan solution, the redox peak currents decreased significantly (Figure 9d) compared to alginate, starch, and gum arabic (Figures 9a,b, and c), indicating that carrageenan binds to the free MB molecules after their displacement from the MB-dsDNA bound complex.

The MB binds reversibly to the bases of the DNA; this occurs through the electrostatic interaction of methylene blue molecules with DNA bases, especially the guanine bases. Moreover, the interaction mechanism between the dye and the DNA is considered a flat penetration of dye molecules due to electrostatic interactions with the phosphate group between pairs of nitrogenous bases of DNA. The interaction of the MB with DNA is electrostatic and reversible, as shown in Figure 10.

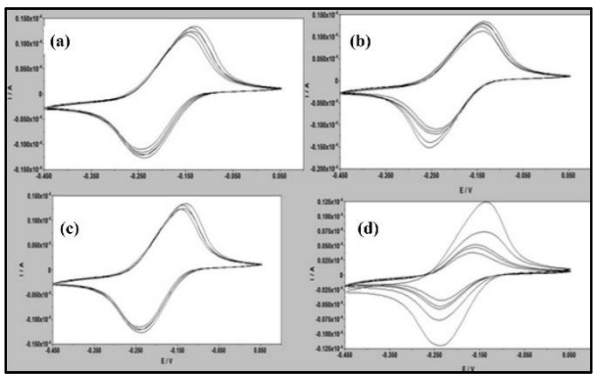

Figure 9: Cyclic voltammetry of dsDNA-SPE in 100 µM methylene blue in Tris-HCl buffer at pH 7. Add polyanion to obtain concentrations of 20, 50, 70, and 100 ppm of polyanion: (a) alginate, (b) starch, (c) Arabic gum, and (d) λ-carrageenan.

The CV detection of competitive interactions between polysaccharides and MB-dsDNA complexes paves several exciting possibilities. This approach offers a sensitive method for analysing carrageenan in food, potentially leading to the development of biosensors for specific carrageenan detection and quantification. Further studies are necessary to explore the influence of different polysaccharides on competitiveness and to optimise electrode surfaces and signal detection strategies for enhanced sensitivity and selectivity [17].

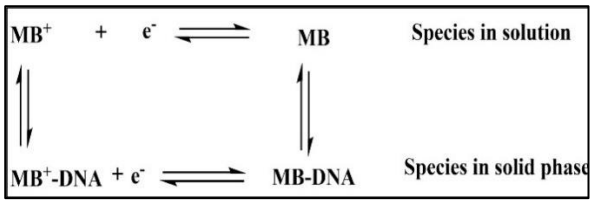

Figure 10: The DNA and Methylene blue interaction process in different species. Reproduced from ref. [33] with permissions from the authors (CC-BY licence). Copyright O. A. Sadik 2001.

4. Conclusion

The present study investigated the competitive interactions between various polyanions and MB-dsDNA complexes using UV-Vis and amperometric methods. Results revealed that dsDNA-MB interactions are reversible and primarily electrostatic, while polyanion-MB interactions exhibit similar reversible electrostatic binding. Carrageenan displayed the strongest competitive interaction with the MB-dsDNA complex, potentially due to its highly charged sulfate ester groups. This superior affinity to other polyanions, including carboxylated polymers, suggests that sulfate significantly enhances competitive binding selectivity. Interestingly, the MB-carrageenan binding affinity followed the order κ-carrageenan > ι-carrageenan > λ-carrageenan, seemingly correlating with their respective sulfate content. Based on these findings, the established competitive interaction between carrageenan and the MB-dsDNA complex holds promise for developing amperometric biosensors for specific carrageenan detection.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

Our research was funded by the Faculty of Science & Technology at Universiti Kebangsaan Malaysia and the Ministry of Education, Malaysia, under the grant FRGS/1/2012/ST01/UKM/01/1. We also acknowledge the assistance of the Department of Fisheries Malaysia and the Malaysian Fisheries Research Institute, who supported us through the grant STGL-007-2010/10.

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