Original Research Article

Converting Methylene Blue into Leucomethylene Blue using Benzalkonium Chloride

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ABSTRACT

Methylene blue (MB) is a cationic dye widely used in biological, chemical, and medical applications. Its reduced form, leucomethylene blue (LMB), is electrically neutral following a proton-coupled electron transfer reaction. Ascorbic acid is commonly used as the electron donor, while hydrochloric acid (HCl) supplies the protons required for reduction. However, the high corrosivity of HCl raises safety and handling concerns. This study investigated benzalkonium chloride (BKC), a quaternary ammonium compound, as a potential non-acidic alternative to HCl in the MB reduction system. Solutions containing 0%, 1%, 5%, 10%, and 20% (v/v) BKC were evaluated. Increasing the BKC concentration generally decreased the final absorbance, with the 20% (v/v) BKC condition producing the lowest final absorbance among the BKC groups. However, the HCl–ascorbic acid control reached a stable absorbance plateau more rapidly than any BKC condition, while the 20% BKC condition required a longer time than the 5% and 10% BKC conditions despite producing the lowest final absorbance. These findings suggest that BKC may serve as a safer alternative to HCl when a greater overall extent of MB reduction is desired, although it does not match the reaction rate of the conventional HCl–ascorbic acid system.

Keywords: Methylene Blue; Leucomethylene Blue; Benzalkonium Chloride; micelle; redox-reaction

INTRODUCTION

Methylene blue (MB) is a cationic dye as well as one of the oldest organic dyes. It has been used for the biological stain, redox-reaction indicator, photosensitizer for generating singlet oxygen, and depressant medicine (1). Leucomethylene blue (LMB) is a reduced form of MB. Since LMB is electrically neutral, it can readily diffuse across cell membranes. Upon oxidation by oxygen, LMB is converted back to methylene blue, producing visible staining of the cell (2). Also, LMB has been used in different research fields; in chemistry, it is used as the oxidation reaction indicator, and in biology, it has been used for the anaerobic bacteria tracker (3).

The reduction of MB proceeds through a proton-coupled electron transfer mechanism (4) MB possesses an extended π-conjugated aromatic ring system. When electron donors–such as ascorbic acid, sodium borohydride, and NADPH–collide and react with MB,the net transfer of electrons is 2; this reduction disrupts the extended conjugation of MB, resulting in the loss of its characteristic visible absorption. The broken pi-conjugation decreases MB to absorb visible-light, and MB becomes colorless. Proton also needs to be transferred to MB. Protonation stabilizes the reduced

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intermediate and facilitates the overall reduction process. Cationic dye, methylene blue, needs 2 electrons to break a double carbon bond in the main ring. While LMB is electrically neutral; therefore, one proton is incorporated into the central ring during reduction, yielding electrically neutral leucomethylene blue (5).

In many studies, hydrochloric acid (HCl) and ascorbic acid have been used in methylene blue (MB) reduction reactions. Ascorbic acid serves as the electron donor, while HCl provides protons required for the reduction reaction. Researchers have long observed that MB reduction proceeds more efficiently under acidic conditions. As a result, HCl has become one of the most commonly used acids in MB reduction systems. In addition to supplying protons, HCl accelerates the reduction reaction (6). Although MB can be reduced by ascorbic acid alone, the addition of HCl supplies extra protons and maintains an acidic environment, which promotes electron transfer from ascorbic acid to MB and increases the reaction rate (7).

However, the use of HCl raises several safety concerns (8). Due to its high corrosivity, HCl can cause severe injury through inhalation, ingestion, and direct contact with the skin or eyes (9). Due to its high solubility in water, concentrated HCl readily releases hydrogen ions. When HCl comes into contact with skin, these hydrogen ions can denature proteins and damage cellular tissues (10). For this reason, we wanted to find an alternative chemical which can convert MB into LMB much more safely for environments.

Benzalkonium chloride (BKC) is a part of the quaternary ammonium compounds and is a positively charged substance (11). It is commonly used as a disinfectant and has a positive nitrogen center with a chloride ion (12). Additionally, it is highly soluble in water which allows it to separate into ions in an aqueous solution (13). Charged regions in the solutions increase molecular collisions which encourages the transfer of electrons and protons. Because of those properties, we hypothesized that BKC would enhance the reduction of methylene blue by facilitating interactions between the reducing agent and MB. After testing multiple experiments, we were able to convert MB into LMB with BKC.

METHODS AND MATERIALS

Materials

In this study, methylene blue (ALDON Innovating Science Lab Grade, 25 g), benzalkonium chloride (UniClean America, 80% concentrate, ASIN B0D1YNV84H), hydrochloric acid (ALDON Innovating Science Lab Grade, 1M), ascorbic acid (Nutricost, Vitamin C Capsules), borosilicate glass scintillation vials (20 mL; GAVKVAY, polypropylene screw cap with aluminum foil liner), and 1000 mL borosilicate glass beaker (Macx Scientific, BKL1K0-002, Griffin low-form) were purchased from Amazon and used without further purification.

Stock Solution

A methylene blue stock solution was prepared by dissolving 20 mg of methylene blue in 700 mL of tap water in a 1000 mL glass beaker. A vitamin C solution was prepared by dissolving two capsules containing 500 mg of ascorbic acid in 500 mL of tap water. Tap water was used to prepare both the methylene blue and ascorbic acid stock solutions because the objective of this study was to evaluate the feasibility of the reaction under readily accessible, practical laboratory conditions.

General Procedure

For each experimental trial, 8.0 mL of the methylene blue solution and 4.0 mL of the vitamin C solution were added sequentially to a 20 mL glass vial. In the study, the total volume of each vial was not equalized. Benzalkonium chloride was added to match the desired concentration: 1% v/v, 5% v/v, 10% v/v, 20% v/v. The solutions were stirred at room temperature for 5 minutes before spectrophotometer analysis.

Control Groups

Control groups used the same methylene blue stock solution and the same volume as the experimental group. For ascorbic-acid-only conditions, 4 mL of ascorbic acid solution was used. For HCl-only condition, 0.227 mL of HCl was added to the vial. 4.0 mL of ascorbic acid stock solution and 0.045 mL of HCl were used for HCl-and-ascorbic-acid condition. The HCl volumes differed between the two control conditions because they were established during preliminary optimization of the experimental protocol. The HCl-only control was intended to evaluate the effect of acidification alone, whereas the HCl–ascorbic acid control was designed to provide sufficient acidity for the reduction reaction while minimizing excess acid in the presence of ascorbic acid.

Data Collection and Analysis

Absorbance measurements were obtained using a visible spectrophotometer (Vernier GDX-SVISPL).

Absorbance was monitored at 665 nm, corresponding to the maximum absorbance peak of methylene blue, allowing direct monitoring of its reduction to leucomethylene blue. Absorbance values were recorded as a function of time (s). Each experimental condition was performed in triplicate, and the reported values represent the mean ± standard deviation (SD) of three independent trials (n = 3). Standard deviations were used to generate error bars, and the relative measurement uncertainty was approximately 3% across all experimental conditions. Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Differences among experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. Statistical significance was defined as $p < 0.05$.

RESULTS

Figure 1 contains different concentrations of benzalkonium chloride and color difference after 5 minutes. The solution was observed for more than 15 minutes to determine whether any additional color change occurred; however, no further change was observed. Because no color change occurred after 15 minutes, the solution was not monitored beyond 5 minutes in subsequent trials. Absorbance versus time (s) was then plotted to examine changes in absorbance over time. Figure 2 shows the absorbance of methylene blue solution at 665nm under different conditions. The HCl-only and ascorbic-acid-only condition had little change in absorbance, throughout the data collected remaining between 1.5 and 1.8 absorbance units. In contrast, all BKC conditions showed decreasing absorbance over time. The amount of decrease of absorbance increased as BKC concentration increased. The 1% v/v BKC condition decreased from approximately 1.6 to 0.95, the 5% v/v and 10%v/v BKC condition decreased to approximately 0.38 and 0.35. The 20% v/v BKC condition showed the largest decrease falling from approximately 1.5 to 0.03 by the end of the experiment. Table 1 summarizes the time required for each condition to reach its minimum absorbance. The HCl–ascorbic acid condition reached its minimum absorbance most rapidly, requiring approximately 174 s. The 10% v/v and 5% v/v BKC conditions required approximately 253 s and 278 s, respectively. The 1% v/v BKC condition did not reach a clear minimum absorbance within the measurement period. Although the 20%v/v BKC condition exhibited the lowest final absorbance, it required a longer period to approach its minimum value.

One-way ANOVA showed that BKC concentration had a significant effect on the final absorbance ($p < 0.001$). Based on Tukey's multiple-comparison test, the 20% BKC group had significantly lower absorbance than the 1% BKC group ($p < 0.01$). However, there was no significant difference between the 5% and 10% BKC groups ($p > 0.05$).

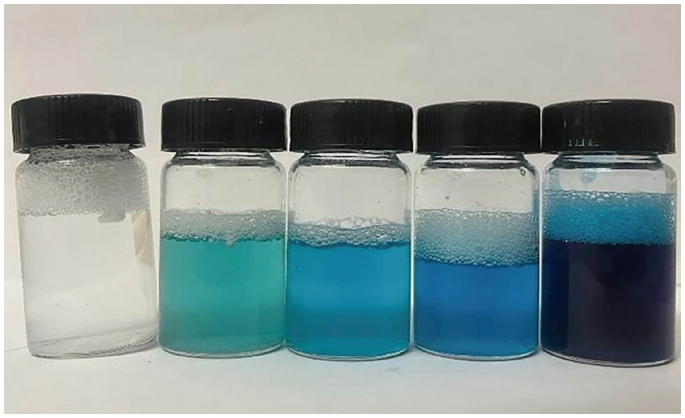

Figure 1. The Picture of 5 methylene blue solution with different concentrations of benzalkonium chloride. *From left (most clear) to right (darkest), the BKC concentrations are 20% v/v, 10% v/v, 5% v/v, 1% v/v, and 0% v/v. Higher BKC concentrations resulted in a greater loss of blue color, indicating a greater extent of methylene blue reduction.*

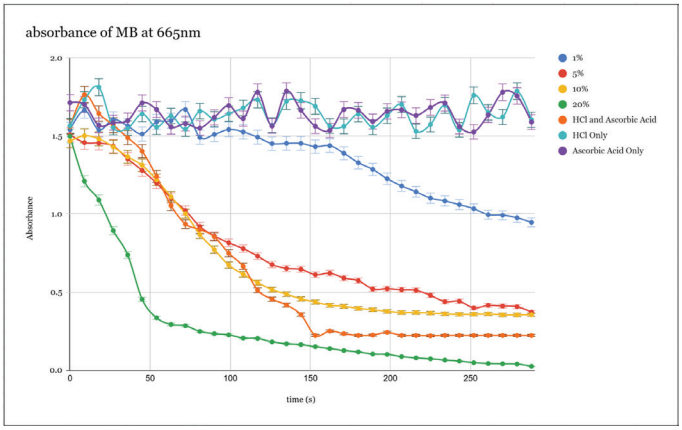

Figure 2. The absorbance after a given time. *Time-dependent absorbance of methylene blue measured at 665 nm under different experimental conditions, including 1%, 5%, 10%, and 20% (v/v) benzalkonium chloride (BKC), HCl only, HCl with ascorbic acid, and ascorbic acid only. Absorbance was recorded as a function of time. Data represent the mean ± standard deviation of three independent trials (n = 3), and error bars represent ± SD.*

Table 1. Time to reach minimum absorbance. *Time required for methylene blue solutions to reach a stable absorbance plateau under different experimental conditions. The plateau time was defined as the first time point after which no further appreciable decrease in absorbance was observed during the measurement period. Values represent the mean ± standard deviation of three independent trials (n = 3).*

Concentration	Time reaching minimum absorbance (s)
HCl and Ascorbic Acid (control)	174 ± 5.22
1% v/v	385 ± 11.55
5% v/v	278 ± 8.34
10% v/v	253 ± 7.59
20% v/v	283 ± 8.49

DISCUSSION

Benzalkonium chloride (BKC) is a cationic surfactant that can form micelles above the critical micelle concentration. In this study, increasing the BKC concentration did not accelerate the reduction of methylene blue compared with the HCl–ascorbic acid control. Instead, higher BKC concentrations produced lower final absorbance values, suggesting a greater overall extent of reduction. One possible explanation is that BKC micelles may have increased the local concentration of MB and ascorbic acid near the micellar interface, thereby increasing the frequency of molecular interactions and electron transfer. As the BKC concentration increased, a greater number of micelles may have been formed, providing more sites for these interactions. This effect could contribute to the lower final absorbance observed at higher BKC concentrations (14, 15).

The present findings are consistent with previous studies on benzalkonium chloride and other surfactant systems. Mahajan et al. reported that benzalkonium chloride forms micelles that promote micellar growth by reducing electrostatic repulsion between surfactant head groups while increasing hydrophobic interactions, resulting in enhanced molecular association. Similar studies have also shown that BKC micellization lowers the critical micelle concentration and facilitates the formation of stable micellar aggregates under favorable solution conditions. These observations agree with the present results, in which increasing BKC concentration produced progressively lower final absorbance values. This suggests that micelle formation may enhance the overall extent of methylene blue reduction by increasing local reactant concentration and stabilizing the reduced species, although it does not necessarily accelerate the reaction compared with the conventional HCl–ascorbic acid system (14, 16).

HCl likely provided an acidic environment in the MB reduction reaction. The acidic environment shifts the redox equilibrium toward the reduced form of MB. Unlike HCl, BKC does not directly influence the acidity of the solution. Instead, BKC may form micelles and stabilize reduced MB, thereby decreasing reoxidation by dissolved oxygen. This may explain why higher BKC concentrations produced lower final absorbance values despite requiring more time to reach a stable absorbance plateau than the HCl–ascorbic acid condition (15).

One limitation of this study is that tap water was used instead of deionized or distilled water to prepare the stock solutions, and the final reaction volume was not kept constant across the different BKC conditions. These factors may have influenced the reduction of kinetics and absorbance measurements. In addition, pH, micelle formation, and dissolved oxygen were not directly measured or controlled. Future studies should address these factors to better isolate the effect of BKC on MB reduction.

CONCLUSION

This study demonstrates that benzalkonium chloride (BKC) has the potential to serve as an alternative component in methylene blue reduction systems. Although increasing the BKC concentration did not accelerate the reduction rate compared with the conventional HCl–ascorbic acid system, higher BKC concentrations produced lower final absorbance values, suggesting a greater overall extent of MB reduction. These findings indicate that BKC may serve as a practical alternative for applications where a more complete reduction is desired while avoiding the use of highly corrosive acids. Future studies should investigate other quaternary ammonium compounds, such as polyDADMAC and Sapamin A, to further understand the reaction mechanism. In addition, examining the effects of different counterions, including sulfate, bromide, fluoride, phosphate, and sulfite, may provide further insight into the role of quaternary ammonium compounds in MB reduction.

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CONFLICT OF INTEREST

The authors: Woojoo Park, Kayla An, Leah Jin, Kelly Kim, Kaityln Lee, and Eline Huh, declare that there are no conflicts of interest regarding the publication of this article.

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