

Study on the Effects of Concentration of Rhodamine B in CPPO Solution on the Chemiluminescence Intensity

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Abstract: In chemiluminescence reactions (CL), the electron jumps to a higher energy level when the fluorescent dye is excited. Dye in an excited state emits light by electrons jumping back to the ground state. In this review, indirect CL was studied with the main focus on the curve of CL intensity due to different concentrations of Rhodamine B (RhB) in bis(2,4,5-trichloro-6-carbopentoxylphenyl) oxalate (CPPO) solution with hydrogen peroxide (PO-CL). The experiments were analyzed directly using a camera and programming because the light produced after the excitation of the dye by CPPO-CL is visible. It was observed experimentally that the CL intensity would increase and then decrease with an increasing concentration of RhB. The resulting data were partially consistent with a Gaussian fit, but after excitation, the trend was more likely to fit consistently with that of logarithmic functions. As the CL intensity was reduced at an RhB concentration of 0.05g/20ml, there is a possibility that the quenching effect exists in the reaction of a high RhB concentration with CPPO. The difference in emission wavelengths and the time to reach maximum brightness are also discussed in this report.

Keywords: Rhodamine B; Concentration; CPPO/H₂O₂; Intensity; Wavelength

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1. Introduction

The earliest natural bioluminescence, that of a glow worm, was recorded in Chinese writing more than 3,000 years ago^[1]. However, artificial chemiluminescence was not reported until the 17th century^[1]. In recent years, the use of CL has become more widespread, such as photodynamic therapy for tumor detection^[2], and quantification of membrane protein shedding on mammalian cells^[3].

Of the many systems, peroxyoxalate is the one most often used. It has a quantum yield of up to 60% and is used extensively in fluorescent rods^[4]. In 2017, Romanyuk, A.V. et al.^[5] discovered that PO-CL response can eliminate tumor cells in medical treatment. The CPPO/H₂O₂ system, one of the most widely used PO-CL with high output photons^[6], then served as an indirect energy donor for the dye in this experiment. The luminescence principle is to produce 1,2-dioxetanedione, a high-energy intermediate, and two molecules of alcohol by the reaction of CPPO and hydrogen peroxide^[1,2,6]. Since 1,2-dioxetanedione was generated, it is likely due to a nucleophilic attack by hydrogen peroxide on the carbon marked with “*” on the benzene ring of CPPO^[1] (**Figure 1**). After bimolecular nucleophilic substitution (SN₂), the high-energy intermediate was used to excite the fuel and release two molecules of CO₂^[1,6,7].

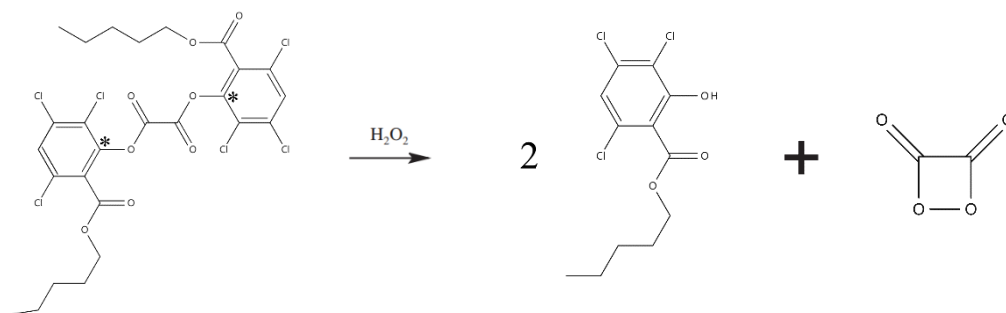


Figure 1. The overall reaction of CPPO and H_2O_2

The fluorescent receptor RhB (shown in **Figure 2**) used in the experiments is a carcinogen and poses a risk to aquatic organisms at high concentrations^[8]. Therefore, it cannot be discharged directly to the sewer, but must first be treated in a particular process, such as using Fenton reagent. RhB appears rosy in water, and can emit bright-red light under certain conditions. The electrons of the excited RhB jump from the first excited singlet state to the ground state and then fluoresce^[9]. Kristoffersen et al.'s study shows that when RhB is excited with light at 850–1020 nm, wavelengths approximately in the range of 440–650 nm will be emitted^[10]. Aromatic heterocyclic compounds like rhodamine may normally undergo rearrangement after photoexcitation, and likely a similar reaction occurs under chemiluminescent conditions.

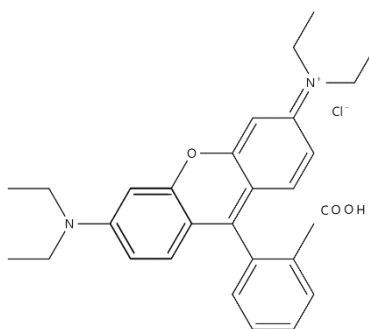


Figure 2. Skeleton formula of RhB

Since the twenty-first century, Chinese studies on CL have gradually increased, but only a few have been translated into English^[1], so I started a discussion on the effect of RhB concentration on the CPPO/ H_2O_2 system based on previous studies. This report emphasizes the impact of the variation of CL light intensity and also speculates on the possibility of experimental results, including quantum leap, fluorescence quenching, and hydroxyl radical oxidation of RhB.

2. Method

2.1. Experimental apparatus and other tools

RhB, $C_{28}H_{31}ClN_2O_3$, Analytical Reagent (AR), Kernel Chemical Reagent Company; CPPO, $C_{26}H_{24}C_{16}O_8$, AR, Shanghai Aladdin Bio-Chem Technology Company; H_2O_2 (30%), AR, Xilong Chemical Company; dibutyl phthalate (DBP), $C_{16}H_{12}O_4$, AR, China National Pharmaceutical Group Corporation; sodium acetate, $CH_3COONa \cdot 3H_2O$, AR, Tianjin Zhiyuan Fine Chemical Plant Company; balance, beakers (50ml), Cylinders(50ml), and so on.

2.2. Preparation

0.01g, 0.02g, 0.03g, 0.04g and 0.05g of RhB, and five portions of 0.05g of sodium acetate were weighed, respectively. A completely light-proof environment with a controlled temperature of 24°C was created. The camera was fixed at one meter above the beaker and was then turned on.

2.3. Experimental procedure

CPPO is easily deliquescent, thus, 1 g of CPPO was measured, then put into a beaker before the start of each group of experiments and the CPPO was kept in a cool place for the rest of the time. 10ml of H₂O₂ and DBP was measured in measuring cylinders A and B, respectively. Liquid B was poured into the measuring cup and stirred until CPPO was dissolved. Solution A, 0.05g of sodium acetate and RhB were added into the beakers in order, and was stirred until well mixed.

2.4. Analysis method

2.4.1. Intensity

The video images were set to the size of 1170 × 1700 and then analyzed by Python to find the average pixel brightness within 13 pixels of the maximum brightness (using a Gaussian blur to obtain the data). The average pixel brightness was analyzed in the maximum brightness area with a radius of 13 pixels by Python.

2.4.2. Wavelength

The standard (red, green, blue) (sRGB) values were read (including the brightness maximum and the surrounding luminescent colors) and converted to Hue values by using Python according to the formula:

$$Hue = \frac{(650 - \text{wavelength}) * 240}{650 - 475}$$

Then, the wavelength was obtained.

2.4.3. Graph

Gaussian fitting, logarithmic fitting, linear fitting and others were used to find the fitted line on the scatter plot that best fits the trend of the scatter and ignore the points that may have significant errors to arrive at a better R² value, which indicates the goodness of fit.

3. Results and discussion

3.1. Variation of time

The variation of chemiluminescence intensity from 0.01 g to 0.05 g of RhB under the reaction with the configured CPPO solutions against time by experiment is shown in **Figure 3**.

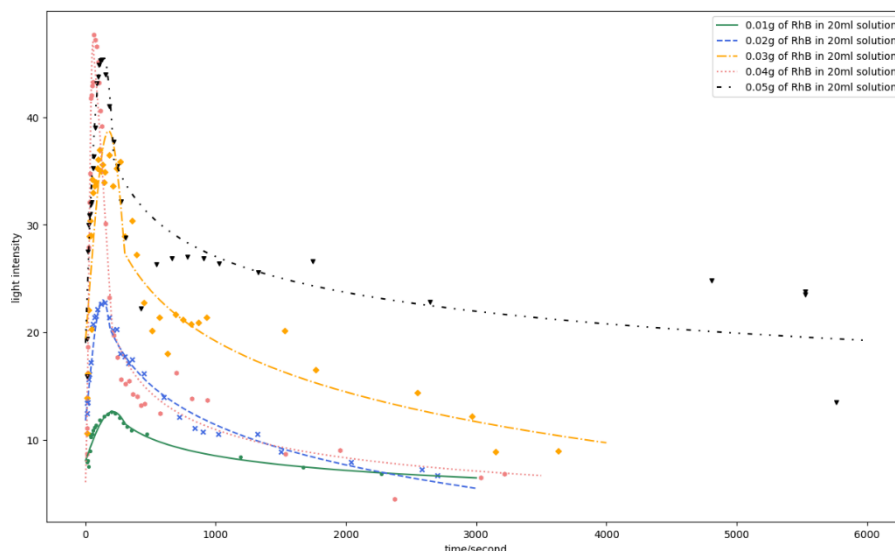


Figure 3. Change in intensity of light emitted by different concentrations of RhB solution

Figure 3 shows that the magnitude of the gradient given by the solution for each concentration is greater before reaching the maximum fluorescence brightness than after. In a previous experimental study, it was shown that after adding imidazole as a catalyst, the brightness of PO-CL varied with time similar to **Figure 3** [5]. The CL lifetime also prolongs with increasing concentrations. An exception was a rapid decrease in brightness when the RhB concentration reaches 0.04 g/20 mL, with the CL lifetime lesser than the one of 0.03 g/20 mL of RhB solution.

In all concentrations, the wavelength of luminescence of the solution visible to the naked eye gradually decreased, approximately from red to orange. **Figure 4** shows the change in color of the solution's luminescence. Since the fluorescence color of RhB differs in different solvents and pHs, like a red shift at pH 2 and a blue shift at pH 6 [11, 12], the color change may be caused by the production of new substances during the experiments that changed the luminescent properties of RhB. The generation of alcohols, as surfactants, due to reaction between CPPO and H₂O₂ may react with the solvent and resulting in a change in color, which will be discussed. Moreover, it is possible that the electron in the higher-energy excited state is initially in the triplet state, so it takes some time to convert from the triplet state to the singlet state and then return to the ground state to emit light. Therefore, this explains the change in the color of the RhB luminescence with time.

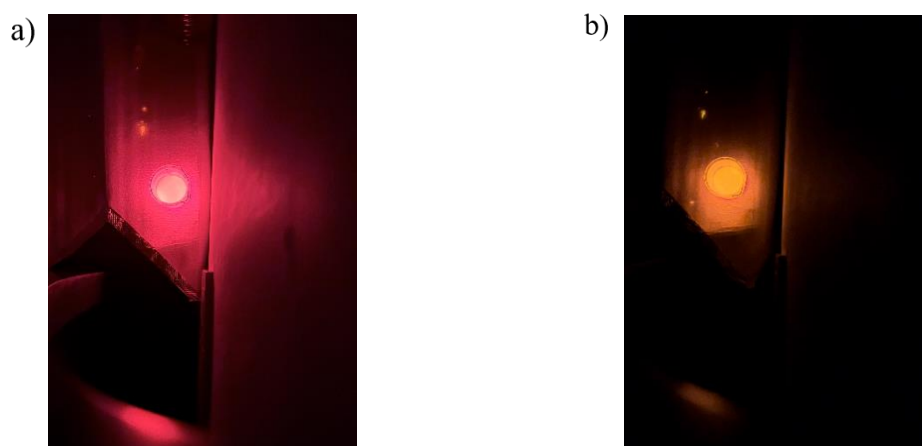


Figure 4. a) Photo of solution with 0.03g RhB that have just started glowing, b) Photo of solution with 0.03g RhB that have been illuminated for about 20 minutes

3.2. Change in maximum wavelength

At the maximum light intensity, the fluorescence color was always rosy red as observed by the naked eye, while the wavelength of the most intense part of the light intensity is not always red. As shown in **Figure 5**, the surrounding color (color of beaker due to reflection) that are the non-luminous parts of liquid and concentration were linearly related with an R^2 of about 0.97302. The trend of the result is consistent with the color change of RhB dissolved in water, based on the reddening of the color of the residue with a higher concentration of RhB after the disappearance of the CL reaction. It may be caused by the increase of unreacted RhB molecules with concentration and dissolution in the waste solution. Hence, when emitting light, the red light obtained from the reaction inside the liquid passes through the liquid more easily. A similar situation was shown in the data of a study done by Min J. et al [13]. They suggested that the red-shift is due to a lower energy level jumps in RhB molecules through π bond interactions to form dimers or polymers, which leads to energy transfers between component molecules [11, 13]. Since intermediates produced CPPO and H_2O_2 was used to provide energy for the dye in this study, which was different from the experiments of Min J. et al. [13], the conclusion above remains to be verified.

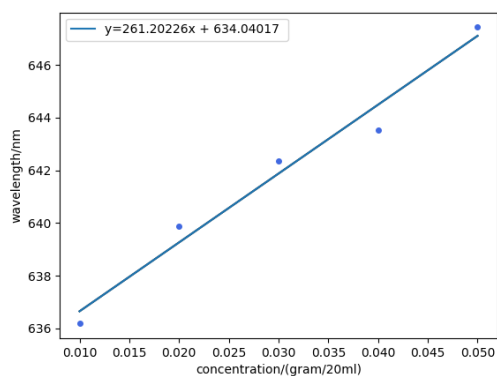


Figure 5. Wavelength of light emitted at maximum brightness (reflections taken from the beaker)

Figure 6 shows a negative correlation between the wavelength at the maximum light intensity emitted from the solution as the RhB concentration increases within the range of 0.01–0.05g/20ml. Disregarding the outlier at the horizontal axis coordinate is 0.02, the best fit line in **Figure 6** has an R^2 of 0.9121.

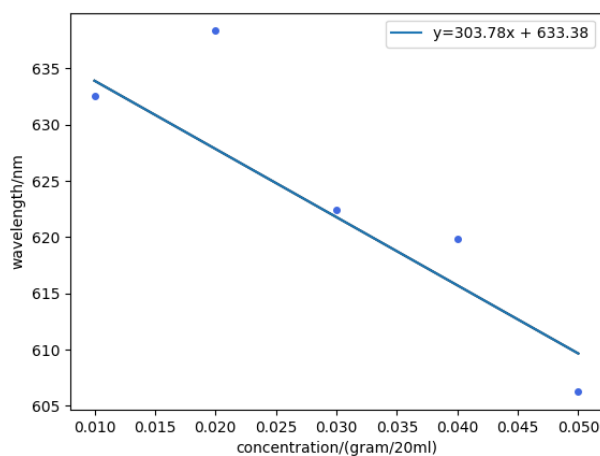


Figure 6. Wavelength of the maximum brightness part in the liquid

When the electron absorbs enough energy to jump to a higher energy level, the wavelength obtained by dropping down to the ground state will be shorter. Theoretically, the substances that provide energy are

of the same mass, and the total energy obtained from each portion of dye should be the same. If we consider the case of increased RhB molecules (previously mentioned), the experimental results and the theory proposed are then contradictory. The change in wavelength might also be correlated with the intensity of CL because the intensity increases when the concentration increases. In the fluorescence reaction, the shorter the emission wavelength (between 500 nm and 650 nm), the higher the brightness [9, 11, 13]. However, the changes in the intensity of the emitted light may only apply to differences in excitation light.

Figure 7 shows that the color of the liquid is not the same as the color of the dispersion in all directions. The resulting wavelength varied depending on the different position of the liquid and the wall of the cup. For example, white light was emitted at approximately the center of the liquid, while red light was observed at the sides of the liquids. The reason for this needs to be further investigated in the future.



Figure 7. Pictures of solutions containing 0.01g/0.03g/0.05g of RhB glowing at maximum brightness

3.3. Change in maximum intensity

Figure 8 shows that all coordinates can be fitted perfectly with a cubic equation in the range of RhB concentrations taken, which shows the CL intensity increasing and then decreasing. At low concentrations, the fluorescence intensity was proportional to the concentration and theoretically what is shown in the **Figure 8** should have been a straight line. However, the slope of the image initially rises and then falls, owing to the possibility of other factors, such as the pH value. Chen and Knutson also pointed out this problem in their report, but it is difficult to define specific best fit lines due to uncertainties in other substances [14].

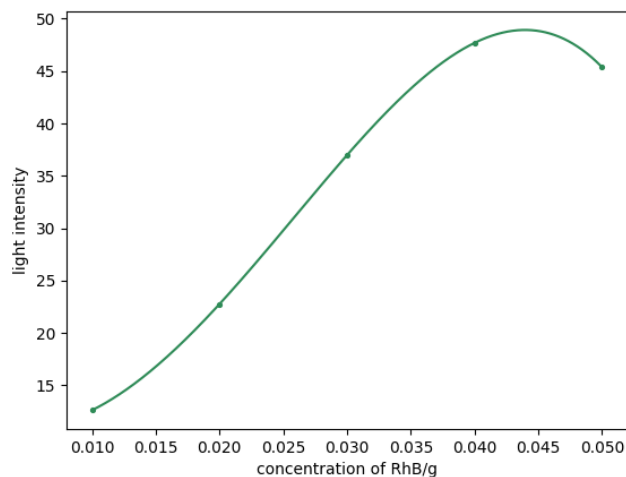


Figure 8. Variation of maximum brightness with concentration

The addition of the dye allows more RhB molecules to collide with the high-energy intermediate, thus better enabling energy transfer from the intermediate to the dye molecule. According to the equation of intensity and Planck-Einstein's law,

$$I = \frac{P}{A}$$
$$E = hf$$

increase in both number and energy of photons will increase the intensity of light. More RhB molecules in the excited state and also according to **Figure 8** (shorter wavelengths of photons have higher energy), higher energy and more photons are emitted. In this aspect, it may give rise to an increase in CL intensity at low concentrations of RhB.

Considering that under the collision of different molecules will cause the generation of quenchers, energy transfer and dynamic quenching may occur. An increase in concentration induces generation of quenchers, which returns the excited molecule to the ground state, thereby reducing the CL intensity. If this trend is followed, the quenching effect can be observed clearly starting at 0.45g/20ml of RhB concentration. In other studies, it has been determined to be able to produce quencher with amines, causing a reduction in fluorescence brightness, and RhB in saturated solution produces a self-quenching effect^[15, 16]. From the remaining liquid after the reaction, the solution was not in the saturated state when 0.01 g of RhB was added, but a saturated solution was formed at 0.02 g. The CL intensity still rises after reaching saturated solution, probably because the quencher is not produced much when there are not many RhB molecules, so the self-extinguishing effect is relatively insignificant. The quenching effect is obvious only after a certain concentration is reached.

3.4. Time to reach maximum brightness

Figure 9 demonstrates the time to reach the maximum fluorescence intensity in different concentrations of RhB solution. The overall trend is downward, except for a increase in time at 0.05 g/20 mL, whereas the R^2 of the best fit line derived from the other four points is 0.7714. An increase in the concentration of the substance increases the rate of reaction. Therefore, the collision between the dye and the high-energy intermediate increases when the concentration of RhB increases. Consequently, the electrons in the excited state of the dye will jump to the ground state earlier and emit light. The production of quenchers also affects the dye in the excited state; therefore, it can be that the continuous production of the quencher makes the fluorescence drop more quickly, which can lead to a shorter time to reach the maximum brightness. The results of 0.05 g/20 mL concentration of RhB solution was not consistent with the prediction, so it is not discussed here.

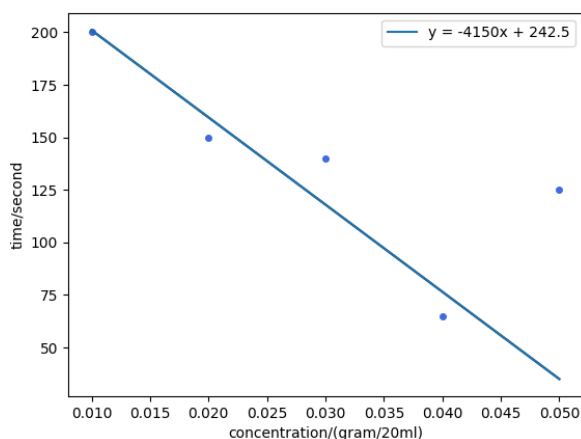
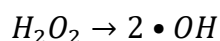


Figure 9. Change in time to reach maximum brightness

3.5. Change in color of residue

In the experimental waste solution, it can be found that with the increase of RhB, the residue color became redder and appeared milky white when there was only 0.01g of RhB whilst the original liquid before the reaction is transparent.

Hydrogen peroxide is unstable, so it will form hydroxyl radicals (as shown below) after receiving heat, ultraviolet light, or other conditions.



In Fenton's reagent, hydroxyl radicals can be generated to oxidize RhB to degrade/decolorize it, and increasing dye concentration also increases the concentration of hydroxyl radicals [17, 18]. Although there was no Fe^{2+} ions to promote the production of hydroxyl groups in the experiments performed in this study, the small amount of hydrogen peroxide decomposition was still sufficient to degrade RhB. The principle is that the hydroxyl radicals attack the central carbon of RhB, destroying its conjugate structure and thus disabling its chromophore, including unsaturated bonds [19]. At the same time, the N-Ethyl group, an auxochrome, is also attacked by hydroxyl radicals, so the destruction of the conjugated structure can also be achieved [20]. As a result, the residue did not appear red after the reaction. However, due to the increase in concentration, the hydroxyls derived from the decomposition of hydrogen peroxide were probably not sufficient to decolorize all of the RhB. Thus, there was some color left. The following **Figure 10** from left to right shows the residual solution of RhB from 0.01g/20mL to 0.03g/20mL concentration respectively, which gradually turns red to the naked eye.

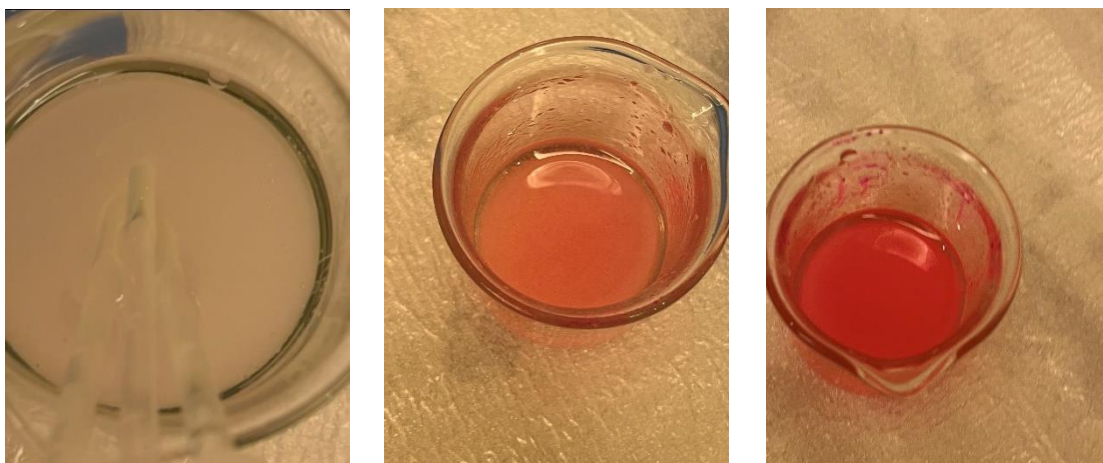


Figure 10. Pictures of waste solutions that adding 0.01g, 0.02g, 0.03g RhB

The generation of the milky solution may be due to the emulsification of the alcohols, produced by the reaction between CPPO and H_2O_2 , and an oily liquid, dibutyl phthalate. This reaction occurs because the resulting alcohol may be a surfactant, in addition to the presence of water in the H_2O_2 .

4. Conclusion

- (1) Fluorescence brightness increases with increasing concentration in the interval from 0.5 g/L to 2 g/L, whilst beyond 2 g/L the CL intensity decreases as the concentration decreases. This may be explained by the fact that an increase in RhB molecules led to an increase in efficiency of the CPPO/ H_2O_2 system, and a larger proportion of the dye was excited and only a small fraction of the energy was dissipated into the surrounding environment. Whereas high concentrations of RhB will react with the liquid to produce a quencher, thus reducing the fluorescence intensity.
- (2) As the concentration increased, the maximum brightness was reached more quickly. A greater amount of substance will shorten the reaction rate due to increased collisions between molecules, thus shortening the reaction time.
- (3) The maximum emission wavelength increased with increasing concentration, whilst the wavelength at the brightest point decreased at decreasing concentrations. This may be attributed to the generation of more aggregates, which results in the occurrence of red-shift and consequently change in luminescence intensity.

5. Limitation

This experiment was not performed in the laboratory, but laboratory conditions were mimicked as much as possible. Nevertheless, the limitations of the study are still significant. The main limitations are divided into three parts, the experimental apparatus, the experimental reagent and the other is the experimental data. As the conditions were limited and the range of wavelengths emitted belongs to a visible spectrum of light, professional experimental equipment such as fluorescence spectroscopy was not used in the experiments. Hence, the measured data and the actual wavelength RhB luminesce range differed, though the overall range was not too different, as they were within about 50 nm. The camera automatically changes the brightness and hue according to the surrounding environment, thus also affecting the calculation of data. Usually, the experiment requires the addition of tert-butanol as a solvent. Tert-butanol is highly toxic, flammable, and not necessary for CL reaction, so this step was omitted in the experiment. There were also some consequences. The CPPO dissolved unevenly and affected the measured fluorescence intensity, for

example, causing the intensity to be high in certain areas. Therefore, a slight incompatibility between water and oil may have occurred in the experiments, thus the usual brightness may have not been achieved after adding tert-butanol.

For the accuracy and reliability of the experimental results, the experiments should have taken more groups of independent variables for the study and include repetitions of the experiments. However, this initiative was not carried out because the experimental process was time-consuming, thus, it led to errors in the experimental data.

This review only included the possible reactions of the CPPO/ H₂O₂ system, and the proposed mechanism is yet to be validated. To increase accuracy, data with possible errors were disregarded in the analysis. In future studies, more professional instruments and standardized experimental procedures should be used to better investigate the patterns of the data.

Disclosure statement

The author declares no conflict of interest.

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